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[GANN, 49, 157~166; Septembers, 1958]

ÜBER DIE HEMMUNG DES ZELLWACHSTUMS MIT AUFTRETEN PATHOLOGISCHER MITOSEFORMEN UND ENTSTEHUNG VON ZWEI- UND MEHRKERNIGEN ZELLEN UNTER DER EINWIRKUNG DES 6-(β-INDOLYLÄTHYL) AMINOPURINS AUF NORMALE ZELLEN UND TUMORZELLEN

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1955 hatten Miller, Skoog, v. Saltza und Strong an der Universität Wisconsin in USA aus gealterter oder autoklavierter Desoxyribonucleinsäure (DNS) einen Faktor isoliert, der an pflanzlichen Zellen Teilung auslöste (1).

Die Zellen wurden in vitro in einem Medium gehalten, in dem ohne Zusatz dieses Faktors nur eine Grössenzunahme, aber keine Teilung stattfindet.

Die als Kinetin bezeichnete Substanz konnte durch Analyse und Synthese als 6-Furfuryl-aminopurin identifiziert werden (2). Diese Arbeit ist insofern interessant, als der beschriebene Faktor nicht nur schon in ganz geringer Konzentration (1γ/L) wirksam ist, sondern auch mit Desoxyadenosin, einer Teilkomponente der DNS, deren Verdoppelung in der Interphase eine Voraussetzung des Mitoseeintritts der Zelle darstellt, in einem nahen strukturellen Zusammenhang steht.

Lettré und Endo testeten das Kinetin an Hühnerfibroblasten und Hela-Zellen in vitro, um festzustellen, ob diese Substanz auch an tierischen Zellen die gleiche Wirkung zeigt, wobei jedoch ein negatives Ergebnis erhalten wurde (3). Wachstumsförderung durch Kinetin ist inzwischen auch bei tierischen Zellen beschrieben worden (4), (5), (6). Ferner wurde versucht, durch Substitution anderer Gruppen statt der Furfurylgruppe des Kinetins, neue Verbindungen darzustellen, die als teilungsauslösende oder mitosehemmende Faktoren in Frage kommen könnten. Eine dieser neuen Verbindungen stellt 6-(β-Indolyläthyl) aminopurin dar, das in der Zelle vielleicht oxydativ zu β-Indolylessigsäure (Wuchshormon der Pflanzen) und Adenin abgebaut werden kann.

Darstellung von 6-(β-Indolyläthyl) aminopurin

Zur Darstellung wurde 6-Methylmercaptopurin mit dem Tryptamin kondensiert, eine Methode, deren Prinzip von Elion, Burgi und Hitchings (7) angegeben worden ist.

0.5 g 6-Methylmercaptopurin und 1.1 g Tryptamin (1 : 2.5 Mol) wurden im Bombenrohr 24 Std. auf 130° erhitzt. Der Inhalt des Bombenrohres wurde mit

heißem Methanol herausgelöst und im Vakuum zur Trockene eingedampft. Der Rückstand konnte aus Methanol-Wasser-Gemisch nach Reinigung mit Tierkohle kristallisiert werden.

Schmp. : 241°. Analyse : $C_{15}H_{14}N_6$.
Ausbeute : 28%. Ber. : C=64.72% H=5.07% N=30.19%
Gef. : C=64.75% H=5.03% H=30.71%

Prufung der Wirksamkeit des 6-(β-Indolyläthyl) aminopurins an normalen Zellen und Krebszellen

Testobjekt und Methodik. Das 6-(β-Indolyläthyl) aminopurin wurde an normalen Zellen (Hühnerfibroblasten) und an Krebszellen (Hela=menschlicher Portiokrebs) untersucht. Die verwendeten Testobjekte zeichnen sich im unbandelten Zustand durch folgende Eigenarten aus:

Hühnerfibroblasten (Abb. 1). Normale unbehandelte Hühnerfibroblastenkulturen bestehen aus einem Verbande gleichmäßig spindelförmiger Zellen. Sie lassen ein vom Zentrum der Kultur ausgehendes radiäres Wachstum erkennen. Die durchschnittliche Mitoserate gut wachsender 24 Stunden alter Kulturen beträgt 3-5%. Dabei finden sich normalerweise keine pathologischen Mitosestadien.

Hela (Abb. 2). Gut wachsende Hela-Kulturen zeigen in ihrem Wachstumssaum die übliche Polymorphie epithelialer maligner Tumoren mit z.T. in Form und Größe vom normalen diploiden Aussehen abweichenden Zellkernen. Öfters kommen recht bizarre Kernbilder vor. Mitosen sind gewöhnlich ziemlich zahlreich vorhanden. Außer normal aussehenden Teilungsstadien kommen in größerer Anzahl pathologische Mitosen vor, z.B. Metaphasen mit Chromosomenaberration, multipolare und polyploide Mitosen gelegentlich auch hochpolyploide Riesenzellen (Abb. 2b). Gerade in diesem Falle zeigt also schon die unbehandelte Kultur besonders reichlich Mitoseanomalien

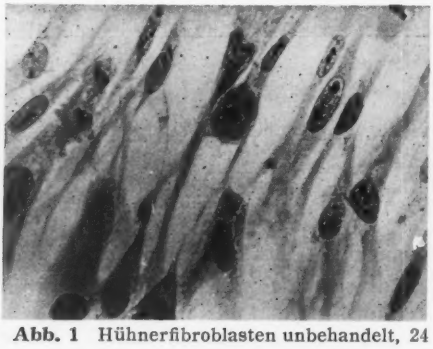

Abb. 1 Hühnerfibroblasten unbehandelt, 24 Std. nach dem Umsetzen der Kultur. Vergr.: 460 fach

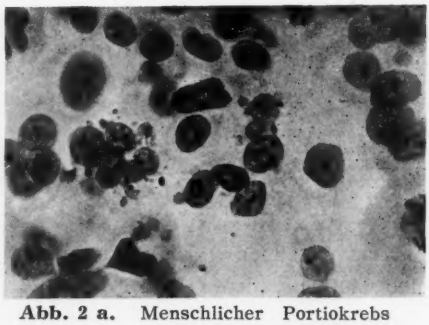

Abb. 2 a. Menschlicher Portiokrebs (Hela) unbehandelt, 48 Std. nach dem Umsetzen der Kultur. Kernpolymorphie. Vergr.: 460 fach

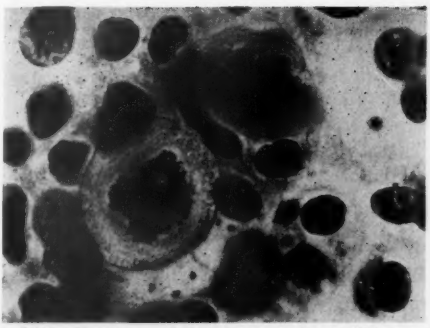

Abb. 2b Menschlicher Portiokrebs (Hela) unbehandelt, 48 Std. nach dem Umsetzen der Kultur Polyploide Mitosen Vergr.: 630 fach

Auf die Methoden und Bedingungen der Gewebezüchtung soll hier im einzelnen nicht eingegangen werden (8), (9), (10). Es wurden Kulturen von der Art verwendet, wie sie am Institut für experimentelle Krebsforschung der Universität Heidelberg laufend hergestellt werden (11), (12).

Die jeweilige Testsubstanz wurde in fester Form dem Nährmedium zugesetzt und zwar entweder in unmittelbarer Nähe der Kultur (n) oder in mittlerer (m) oder weiterer (w) Entfernung von der Kultur.

Für die Untersuchungen an Hühnerfibroblasten wurden die Kulturen in der Mehrzahl der Fälle beim Umsetzen gleichzeitig mit den Kristallen der zu prüfenden Substanz versetzt. In vier Fällen wurde die Substanz 48 Stunden alten Kulturen appliziert.

Die Krebszell-Kulturen wurden dagegen zunächst von Carrel-Flaschen oder Rollertubes nach der Ringwall-Methode auf ein Deckglas umgesetzt und 48 bis 72 Stunden im Brutschrank gehalten, bis sie ein gutes Wachstum erkennen ließen. Erst dann wurde die jeweilige Testsubstanz zugesetzt.

Das 6-(ß-Indolyläthyl) aminopurin wurde nicht nur in fester Form, sondern auch in Lösung getestet. Dazu wurde eine Auflösung der Substanz in Gey'scher Salzlösung zu gleichen Teilen mit Hühnerplasma und einem Gemisch aus Placentaserum, Embryonalextrakt und Salzlösung im Verhältnis (2 : 1 : 2) versetzt und tropfenweise den Gewebekulturen zugesetzt. In Kontrollversuchen erfolgte anstatt der Substanzzugabe ein Zusatz einer indifferenten Salzlösung.

Nach der Applikation der zu untersuchenden Substanz wurden die Kulturen im Brutschrank bei einer Temperatur von 38.5°C (Fibroblasten) bzw. 36.5°C (Tumorgewebe) aufbewahrt, nach verschiedenen Zeitintervallen von 12 bis 72 Stunden nach Bouin fixiert, mit Hilfe der Hämalaun-Methode getärbt und dann cytologisch ausgewertet. Die Auszählung der Mitosen erfolgte in der übersichtlichen äussersten Wachstumszone. Es wurden ca. 1000 Zellen je Präparat ausgezählt. Die Mitoserate wurde in % der Gesamtzahl der gezählten Zellen ausgedrückt. Parallel zu den Substanz-Versuchsreihen liefen unbehandelte Kontrollversuche mit Kulturen aus dem gleichen Muttergewebe wie die behandelten Versuchskulturen.

Ergebnisse

In den Testversuchen mit 6-(ß-Indolyläthyl) aminopurin in kristalliner oder gelöster Form an Kulturen normaler Zellen und Krebszellen ergaben sich folgende Befunde, die für Fibroblasten zur besseren Übersicht in Tabellenform dargestellt sind. (Tab. 1-3).

Zusammenfassend ergibt sich folgendes Bild: Nach der Einwirkung von 6-(ß-Indolyläthyl) aminopurin in fester Form auf Hühnerfibroblasten (Tab. 1) lässt sich in allen Versuchen eine gegenüber den gleichzeitig durchgeführten Kontrollen eindeutige Mitosehemmung beobachten. Im cytologischen Bild zeigt sich eine deutlich ausgeprägte Erhöhung der Mitosezahlen, die hervorgerufen wird durch eine Anhäufung arretierter, zum grossen Teil stark verklumpter Metaphasen mit einzelnen oder mehreren Polchromosomen und Drei-Gruppen-Metaphasen ("métaphases à trois groupes" nach Parmentier und Dustin 1948) (13) vor (Abb. 4, 5). Die Ruhezellen sind häufig verbreitert. Weiterhin finden sich, in besonderem Masse nach einer längeren Applikationsdauer, in grosser Zahl zwei-und mehrkernige Zellen (Tab. 1 und Abb. 6) sowie Zellen mit bizarr geformten

Tab. 1: 6-(β-Indolyläthyl) aminopurin in fester Form getestet an Hühnerfibroblasten.

Versuchskulturen					Kontrollkulturen	
Zeit der Substanz-einwirkung in Stunden	Substanz-entfernung von der Kultur	Anzahl der Versuchs-Kulturen	Mitosen % (Mittelwert)	Zwei-und mehrkernige Zellen % (Mittelwert)	Anzahl der Kontroll-Kulturen	Mitosen % (Mittelwert)
12	nah	1	10.65	1.42	3	2.25
	mittel	2	6.34	1.13		
16	nah	2	12.72	1.95	3	3.72
	mittel	1	9.63	4.19		
20	nah	7	9.20	12.20	10	3.49
	mittel	3	7.51	8.98		
24	nah	8*	10.99	8.73	10	3.60
	mittel	8	10.58	10.77		
28-31	nah-mittel	5	12.56	18.55	5	3.53
34	nah	2	6.51	24.04	2	4.26
48	nah	6	6.57	25.90	9	2.44
	mittel	6	5.84	18.21		
	weit	1	6.60	10.00		
72	nah	2	3.90	29.94	6	0.51
	mittel	5	2.18	22.01		

* Von den 8 Kulturen (24 Std. n) wurden zwei- und mehrkernige Zellen nur bei 4 Kulturen ausgezählt.

Kernen und zahlreichen Nucleolen (Abb. 8), die aus pathologisch veränderten Metaphasen hervorgehen dürften. Gelegentlich wurden zweikernige Mitosen beobachtet (Abb. 7). Auffallig ist, dass mit längerer Behandlungsdauer die Zunahme der mehrkernigen Zellen einem Absinken der Mitoserate parallel geht.

Bei der Behandlung 48 Stunden alter Fibroblastenkulturen mit 6-(ß-Indolyläthyl) aminopurin (Tab. 2) lässt sich nach 24 stündiger Einwirkungsdauer keine so eindeutige Mitosehemmung mehr beobachten wie im vorigen Versuch, obwohl die Zahl der Mitosen im Vergleich mit dem Mitosewert der Kontroll-Kulturen immer noch erhöht ist und insbesondere die zwei- bis vielkernigen Zellen noch deutlich, z.T. erheblich vermehrt sind. Es finden sich Verbreiterung und Kernpolymorphie der Interphasezellen, arretierte, verklumpte, oder anderweitig pathologisch veränderte Metaphasen mit Chromosomenaberration in Form von einzelnen oder mehreren Polchromosomen sowie gelegentlich "métaphases à trois groupes."

Tab. 2: 6-(β-Indolyläthyl) aminopurin getestet an 48 Stunden alten Hühnerfibroblasten.

Versuchskulturen					Kontrollkulturen	
Zeit der Substanzeinwirkung in Stunden	Substanzentfernung von der Kultur	Anzahl der Versuchskulturen	Mitosen %	Zwei- und mehrkernige Zellen %	Anzahl der Kontrollkulturen	Mitosen %
24	nah-mittel	6	0.70	3.30	6	0.00
			1.65	7.51		0.10
			1.29	4.71		0.38
			1.60	8.35		0.10
			2.00	15.60		0.10
			1.70	11.65		0.20

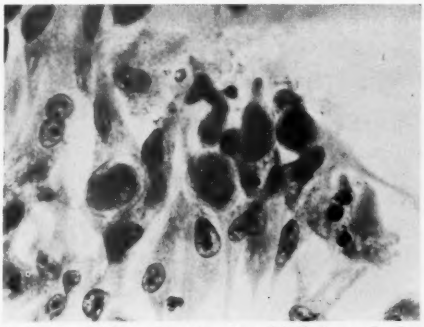

Abb. 3 Hühnerfibroblasten 24 Std. nach der Applikation von 6-(β-Indolyläthyl) aminopurin. Chromosomenverklumpung
Vergr.: 460 fach

Abb. 4 Hühnerfibroblasten 16 Std. nach der Applikation von 6-(β-Indolyläthyl) aminopurin. Mehrere Mitosen mit Polchromosomen bzw. "métaphases à trois groupes"
Vergr.: 460 fach

Abb. Hühnerfibroblasten 16 Std. nach der Applikation von 6-(β-Indolyläthyl) aminopurin. "Métaphase à trois groupes". Vergr.: 920 fach

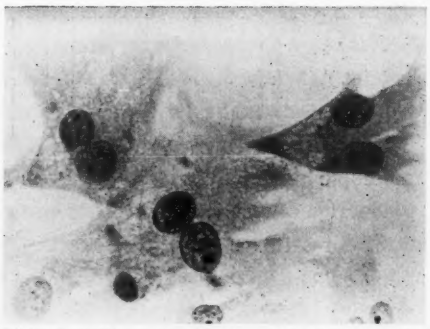

Abb. 6 Hühnerfibroblasten 48 Std. nach der Applikation von 6-(β-Indolyläthyl) aminopurin. Zweikernige Zellen. Vergr.: 460 fach

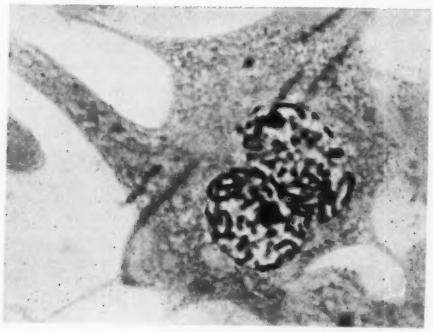

Abb. 7 Hühnerfibroblasten 48 Std. nach der Applikation von 6-(β-Indolyläthyl) aminopurin. Zweikernige Zellen in Prophase Vergr.: 1260 fach

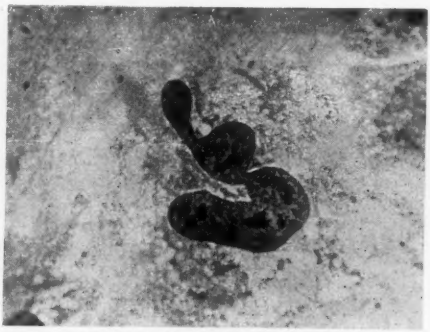

Abb. 8 Hühnerfibroblasten 72 Std. nach der Applikation von 6-(β-Indolyäthyl) aminopurin. Bizarr geformter Zellkern mit vermehrten Nucleolen. Vergr.: 630 fach

Tab. 3: 6-(β-Indolyläthyl) aminopurin in Lösung getestet an Hühnerfibroblasten.

	Versuchskulturen				Kontrollkulturen	
Zeit der Substanzeinwirkung in Stunden	Konzentration der Versuchslösung	Anzahl der Versuchskulturen	Mitosen % (Mittelwert)	Zwei- und mehrkernige Zellen % (Mittelwert)	Anzahl der Kontrollkulturen	Mitosen % (Mittelwert)
26	60 γ/ml	2	12.10	4.20	2	3.22
	30 γ/ml	3	8.31	16.09	1	3.47
	12 γ/ml	3	9.32	9.25	—	—
48	1.2 γ/ml	3	1.38	vereinzeltes vorkommen	3	2.56

Auch bei Verwendung von 6-(β-Indolyläthyl) aminopurin in Lösungsform (Tab. 3) läßt sich in der Mitosezahl und in der Zahl der zwei- und mehrkernigen Zellen

ein Befund erheben, der dem bei Verwendung fester Substanz in allen wesentlichen Punkten entspricht. Erst bei einer Konzentration von 1γ/ml läßt sich keine eindeutige Substanzwirkung mehr nachweisen.

6-(β-Indolyläthyl) aminopurin getestet an Hela

Die Kristalle von 6-(β-Indolyläthyl) aminopurin wurden den Versuchskulturen nur einseitig zugesetzt. Ein Teil der Kulturen wurde 13 Stunden, ein anderer Teil 24 Stunden nach Einwirkung der Substanz fixiert. Es zeigte sich ein überraschender Befund: Es wurden massenhaft Mitosen gefunden, die für eine Mitosehemmung, bzw. Verzögerung des Mitoseablaufs sprechen. Vorwiegend handelt es sich um arretierte Metaphasen mit relativ dichter Chromosomenlagerung, ohne daß eine so ausgesprochene Verklumpung wie nach Colchicin zu erkennen war. (Abb. 9a, 9b). Besonders auffällig war eine mehr oder weniger große Anzahl Metaphasen mit charakteristischer Chromosomenanordnung, wie sie weniger zahlreich auch bei den entsprechend behandelten Fibroblastenkulturen vorkamen: einzelne Chromosomen oder Chromosomengruppen lagen außerhalb der normal aussehenden Äquatorialplatte (Abb. 10), sehr häufig an beiden Spindelpolen, wie sie von Parmentier und Dustin 1948 als "métaphases à trois groupes" beschrieben wurden (Abb. 11, 12, 13). Anaphasen und Telophasen waren nicht festzustellen. Im übrigen wurden atypische Metaphasen gefunden, wie sie auch in Hela-Kontrollen vorkommen.

Im Vergleich zu den 24 Stunden-Kulturen zeigten Kulturen, die 48 Stunden unter dem Einfluß der Substanz standen, auffällig wenig Mitosen.

Besprechung der Ergebnisse

Die Untersuchung der Wirksamkeit des 6-(β-Indolyläthyl) aminopurins an tieri-

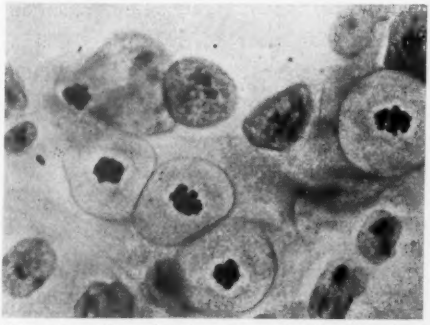

Abb. 9a Menschlicher Portiokrebs (Hela) 24 Std. nach der Applikation von 6-(β-Indolyläthyl) aminopurin. Mitosearretierung in Metaphase. Vergr.: 630 fach

Abb. 9b Menschlicher Portiokrebs (Hela) 24 Std. nach der Applikation von 6-(β-Indolyläthyl) aminopurin Arretierte Metaphasen Vergr.: 920 fach

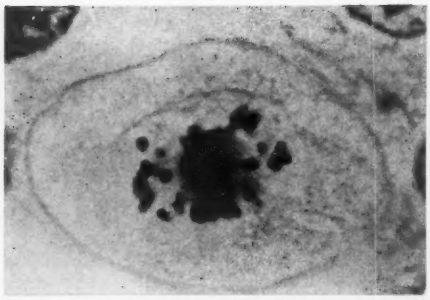

Abb. 10 Menschlicher Portiokrebs (Hela) 24 Std. nach der Applikation von 6-(β-Indolyläthyl) aminopurin. Metaphase mit Chromosomenaberration. Vergr.: 1260 fach

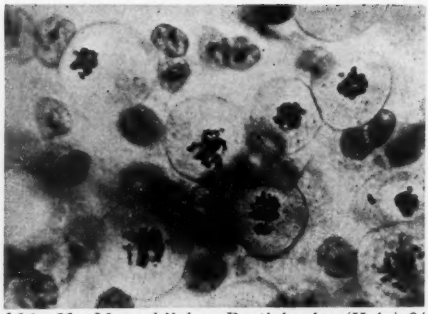

Abb. 11 Menschlicher Portiokrebs (Hela) 24 Std. nach der Applikation von. 6-(β-Indolyläthyl) aminopurin. Métaphases à trois groupes. Vergr.: 630 fach

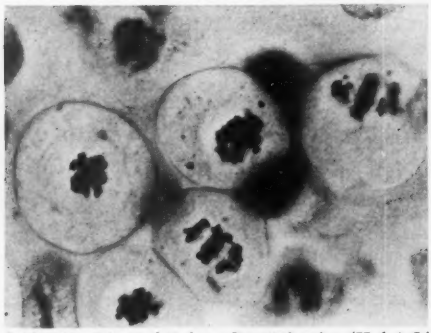

Abb. 12 Menschlicher Portiokrebs (Hela) 24 Std. nach der Applikation von 6-(β-Indolyläthyl) aminopurin. Métaphases à trois groupes. Vergr.: 920 fach

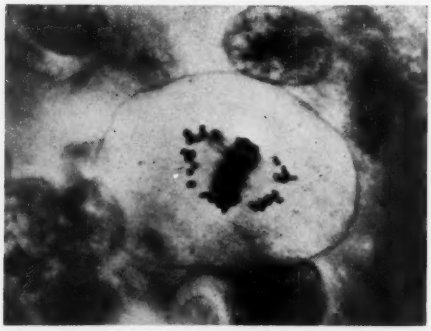

Abb. 13 Menschlicher Portiokrebs (Hela) 24 Std. nach der Applikation von 6-(β-Indolyläthyl) aminopurin. Métaphases à trois groupes. Vergr.: 1260 fach

schen Geweben (Fibroblasten und Tumoren in vitro) ergab folgende überraschende Befunde: Bei der Verwendung dieser Verbindung in fester Form und als Lösung (Dosierung über 10γ/ml) fand sich sowohl an Fibroblasten-Kulturen, denen die Substanz bereits beim Umsetzen zugesetzt wurde, als auch an Kulturen, die erst nach 48 stündigem Wachstum mit der Substanz behandelt wurden, eine deutliche Mitosehemmung. Dabei zeichnete sich das cytologische Bild durch eine Anhäufung arretierter, z.T. verklumpter oder anderweitig pathologisch veränderter Metaphasen mit einzeln oder gruppenweise aberrierten Chromosomen aus, in ganz besonderem Maße war aber die Zahl der zwei- bis vielkernigen Zellen erhöht. Auffällig waren außerdem Interphasezellen mit bizarr geformten Kernen und zahlreichen Nucleolen bei Fibroblastengewebe.

Eine ebenso deutliche Hemmwirkung auf das Teilungsgeschehen wie für Fibroblasten beschrieben, konnte auch an Tumoren beobachtet werden. Auch hier

fand sich bei fast völligem Verschwinden von Ana- und Telophasen eine eindrucksvolle Arretierung der Mitosen im Metaphasestadium. Die Chromosomen der gleichzeitig pathologisch veränderten Metaphasen wiesen jedoch nicht eine Zerstreuung oder Verklumpung (wie z.B. nach Colchicin) auf, sondern ließen häufiger als am Fibroblastengewebe bei allen verwendeten Tumorarten eine eigenartige und für diese Substanz wohl typische Anordnung in drei Gruppen erkennen. Diese Chromosomengruppierung ist erstmalig 1948 von Parmentier und Dustin (13) beschrieben und als "métaphase à trois groupes" bezeichnet worden. Die beschriebenen Befunde beanspruchen deshalb ein besonderes Interesse, weil es sich bei 6-(β-Indolyläthyl) aminopurin, wie in der Einleitung dargelegt wurde, um eine Verbindung handelt, die bei einer oxydativen Spaltung in Adenin und Heteroauxin zerfällt. Adenin selbst hat nach Hughes (14) eine verwandte Wirkung hinsichtlich der Beschleunigung der Rekonstruktion der Ruhekerne, jedoch erst bei einer wesentlich höheren Dosis von 500γ/ml. Das Heteroauxin ist für entsprechende Untersuchungen an pflanzlichen Zellen von großem Interesse.

Eine ähnlich hemmende Wirkung auf das Teilungsgeschehen der Zelle, wie wir sie bei 6-(β-Indolyläthyl) aminopurin beobachteten, liegt auch bei Colchicin vor. Beide Substanzen führen bei Anwendung höherer Konzentrationen zu einer Arretierung im Metaphasestadium, wodurch es besonders bei Colchicin zu einer sehr starken Mitoseanhäufung kommt. Während aber Colchicin infolge Spindelschädigung zu einer Chromosomenverklumpung und -zusammenballung führt, wodurch die Trennung der Chromosomen und damit die Bildung der Tochterkerne gestört wird, lassen sich nach Einwirkung von 6-(β-Indolyläthyl) aminopurin vor allem Chromosomenverlagerungen in Form einzelner aberrierter Chromosomen (Polchromosomen) und typischer 3-Gruppen-Metaphasen beobachten. Durch diese Verlagerungen, die je nach der angewandten Dosis mit einer Verlangsamung oder Hemmung der Chromosomentrennung bei erhaltener Fähigkeit zur Kernrekonstruktion aber ohne Cytoplasmateilung einhergehen, fände die nach 6-(β-Indolyläthyl) aminopurin in besonderem Maße zu beobachtende Mehrkernigkeit der Zellen ihre Erklärung.

Zusammenfassung

6-(β-Indolyläthyl) aminopurin wurde dargestellt, dessen Wirkung auf normale Zellen (Hühnerfibroblasten) und Tumorzellen (Hela) an Gewebekulturen getestet und cytologisch untersucht.

Es zeigte sich eine etwa gleichmäßig starke Hemmung des Zellwachstums mit Auftreten pathologischer Mitoseformen und eine deutlich Anhäufung von zwei- und mehrkernigen Zellen.

Herrn Professor Dr. H. Lettré bin ich für die freundliche Überlassung des Themas, die

Anregung und Förderung der Untersuchungen zu ganz besonderem Dank verpflichtet.

Frau Dr. R. Lettré und Fräulein Dr. W. Siebs danke ich herzlich für die freundliche Unterstützung, Herrn A. Mayer für die Hilfe bei Herstellung der Mikrofotographien.

Der Alexander von Humboldt-Stiftung, die mir den Studienaufenthalt in Deutschland ermöglichte, gilt mein besonderer Dank.

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要　旨

6-(β-Indolyläthyl) aminopurin により正常および腫瘍細胞に観察された病的分裂像の出現，二核並びに多核細胞形成を伴える細胞増殖阻止効果について

遠藤英也 (ハイデルベルグ大学実験癌研究所：所長　ハンス　レトレ教授)

植物細胞に対する分裂促進素 Kinetin を基礎としてそのフルフリール基を他の種々のグループで置換して数種の新誘導体を合成しより強力な分裂促進能または反対に分裂阻止効果を期待した。その一つ 6-(β-Indolyläthyl) aminopurin は組織培養上で正常細胞として用いた鶏胚心の繊維芽細胞および癌細胞として用いた HeLa 細胞に対し 10γ/ml 以上の濃度で細胞分裂を抑制し同時に多数の2核および多核細胞の出現を見た。また該物質は分裂期の metaphase に作用しこの物質に特異的ともいうべき "métaphase à trois groupes" を，形成し，その他種々の病的分裂像が観察された。

[GANN, 49, 167~170; September, 1958]

TETRAHYDROKINETIN

(Plates XIX and XX)

HIDEYA ENDO
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and KAZUO NITTA
(The National Institute of Health of Japan, Tokyo)

The interphase of the cell is characterized by a great synthetic ability of cell components, especially through the doubling of the desoxyribonucleic acid content[1,2]. Although the doubling of the amount of DNA, and also the synthesis of protein, ribonucleic acid, etc., are provisions for mitosis, another factor seems to be needed in order to permit the cell to enter into mitosis, as has been postulated by Mazia.[3,4] Little is known concerning this point, however.

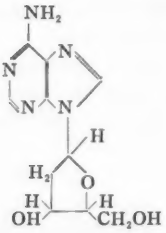
Of great interest in this connection is kinetin, a distinct mitosis stimulating factor for plant cells, which was first discovered in 1955 by Miller, Skoog and associates[5] and was identified as 6-furfurylaminopurine[6].

Lettré[7] assumed it to be possible that either desoxyadenosine [I] present in the cell as a precursor of nucleic acid undergoes a transition so that the primary alcohol group of desoxyribose reacts with 6-amino group of adenine under splitting off of water, or a similar reaction is induced between two desoxyadeonosine molecules, instead of intramolecular transition, with the result that 6-(3′,5′-dioxytetrahydrofurfuryl) aminopurine [II] is produced, and then 6-furfurylaminopurine, i. e. kinetin [III] by further splitting oft of two Mol of water from furanose system. From these possibilities one may make the following assumptions as regards the mechanism of the induction of mitosis:

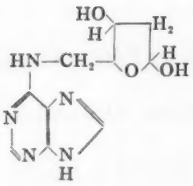
a). The consumption of [I] for the doubling of the amount of DNA in the course of reaction in interphase.

b). The secondary, accompanying reaction of a small percentage of [I] upon [II] towards kinetin [III], through the presence of which the cell can enter into the mitosis. This reaction can take place only when DNA is no longer formed.

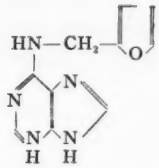
Based on this very interesting and attractive hypothesis of Lettré, we first synthesized 6-tetrahydrofurfurylaminopurine (tetrahydrokinetin), as a model of the hypothetical intermediate substance described above [II]. Much to our interest, this substance was found to inhibit the growth *in vitro* of HeLa cells and our findings will be reported in this paper. Kinetin has no effect on animal cells in tissue culture, as already well established.[8]



〔I〕



〔II〕



〔III〕

Preparation of Tetrahydrokinetin

A mixture of 1.7g (0.011 mol) 6-chlorpurine, 3.4 g (0.036 mol) tetrahydrofurfurylamine and 5 ml methylcellusolve was heated for 2 hours under reflux. After cooling the crystals which appeared were recovered by filtration, washed with a small amount of water and then of acetone, and were recrystallized from methanol.

M. p.: 187°	Yield: 1.23 g (51%)			
$C_{10}H_{12}N_5O$	Calculated:	C 54.78	H 5.98	
	Found:	C 54.62	H 5.76	

The crystal was dissolved in methanol and then ether saturated with dry HCl added. After the solvent was distilled off the crystal was recrystallized from etherial methanol.

M. p.: 210°	Yield: 1.00 g (69.7%)			
$C_{10}H_{14}N_5OCl$	Calculated:	C 46.97	H 5.52	N 27.39
	Found:	C 46.68	H 5.10	N 27.67

Effect of Tetrahydrokinetin on HeLa Cells

For the examination of the effect of tetrahydrokinetin on animal cells, a tissue culture method using strain HeLa cells was employed. The strain HeLa used was that which had been obtained from Dr. Takemori of the National Institute of Health, Tokyo, and maintained by the present authors since May 1954. As the procedure, use was made of the principle of Nitta's method[9]. The outline of the procedure will be briefly described here. A healthy seven day old culture HeLa was processed with 0.02% sodium versenate solution to give a homogenous cell suspension. Following a centrifugation of the suspension, the sedimented cells were resuspended in an aliquot of the fluid medium, which consisted of 8 parts of lactalbumin-yeast extract saline and 2 parts of human adult serum. The lactalbumin-yeast extract saline was prepared by the addition of lactalbumin hydrolysate, yeast extract and dextrose to Earle's saline at concentrations of 0.5%, 0.1% and 0.45% For the replicate culture, 0.95 ml of the cell suspension

thus prepared was poured into a small square tube with a graduated pipette. The concentration of the inoculum was approximately 10^5 cells/ml. After three day incubation, 0.05 ml of the solution of tetrahydrokinetin was added to the healthy cultures. A daily macro- and microscopical observation was performed for the three successive days. On the other hand, the pattern of the cell number in those culture series which had been processed in the same manner was pursued by daily nuclear counting using Sanford-Katsuta's method[10,11]. The test solution to be added was prepared by dissolving tetrahydrokinetin hydrochloride in redistilled water and was adjusted to pH 7.2. Two-fold dilution series was employed. No precipitation was observed in any solution of tetrahydrokinetin.

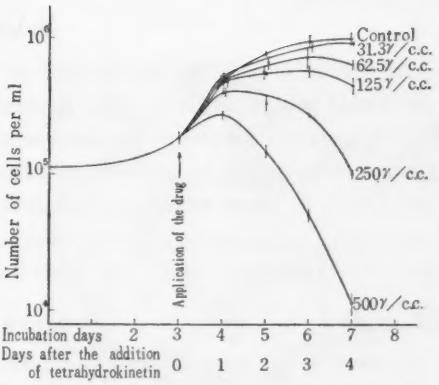


Chart 1. The pattern of the changes in cell number in the cultures exposed to tetrahy drokinetin.

The results are shown in Table 1, and chart 1, and are illustrated in Plates XIX and XX.

Table 1. The change in the cell number following the addition of tetrahydrokinetin to the cultures.

Concentration of Tetrahydrokinetin (γ/c.c.)	Cell number ($\times 10^3$)				
	Prior	1 day	2 days	3 days	4 days
500	189±20	273±17	147±16	50± 11	11± 2
250		400±28	327±36	272± 4	98± 7
125		475±38	541±10	564± 54	439± 59
62.5		489±38	559±60	716±126	626± 51
31.3		514±28	654±66	851± 71	902± 32
Control		468±79	760±35	932± 85	968±101

Discussion

In 1956, Lettré and Endo[8] demonstrated, with the aid of tissue culture method, that kinetin produces no effect on cell proliferation, either normal (chicken fibroblasts) or malignant cells (HeLa). In marked contrast to this, tetrahydrokinetin, which has only four more hydrogen atoms than kinetin, showed a distinct inhibition of the growth of HeLa cells. This interesting fact will be subjected to further analysis.

Summary

As the side path of the process in which nucleic acid is synthesized from nucleotide pool, a pathway from desoxyadenosin to kinetin is supposed. From this speculation tetrahydrokinetin was synthesized as a model of the hypothetical intermediate. This substance was found to markedly inhibit the growth of HeLa cells in tissue culture, although kinetin is known to have no effect.

We wish to express our special thanks to Professor Hans Lettré for his kind support of this work. Our thanks are also due to the Laboratory of the Kowa-Kagaku Co., Tokyo.

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要　　旨

テトラヒドロキネチン

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ヌクレオチドプールから核酸が合成されて行く main path のわき道としてヌクレオチドからキネチンえの系路を想定しその想定中間体のモデルとしてテトラヒドロキネチンを合成した興味深いことにはキネチン自体は動物細胞に対し組織培養上何等の作用をも示さないのに対しこのものは HeLa 細胞に対し著しい増殖抑制作用を示した。　(文部省科学研究費による)

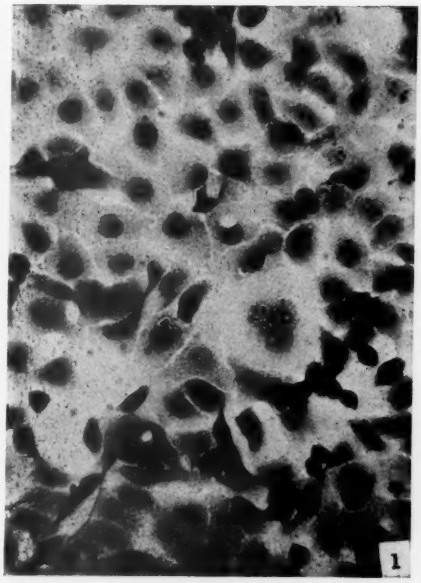

Fig. 1. 24 hrs. control

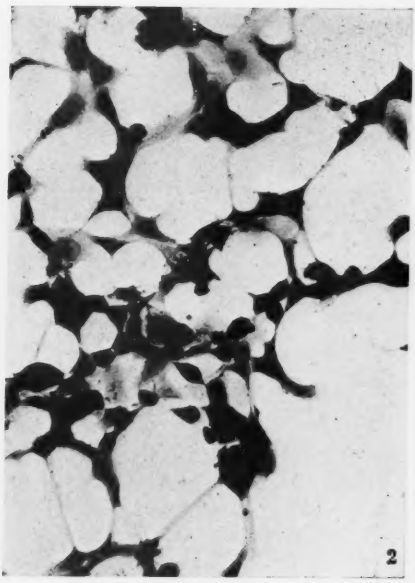

Fig. 2. 24 hrs. after the application of tetrahydrokinetin at the concentraton of 1000 γ/ml

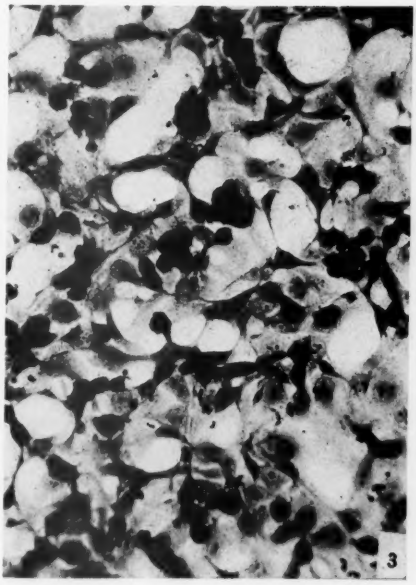

Fig. 3. 24 hrs. after the application of tetrahydrokinetin at the concentration of 500 γ/ml

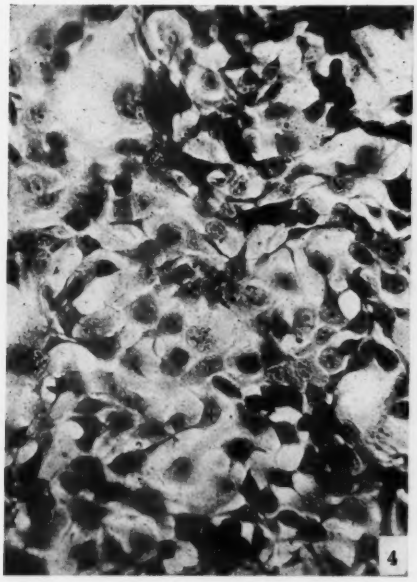

Fig. 4. 24 hrs. after the application fo tetrahydrokinetin at the concentration of 250 γ/ml

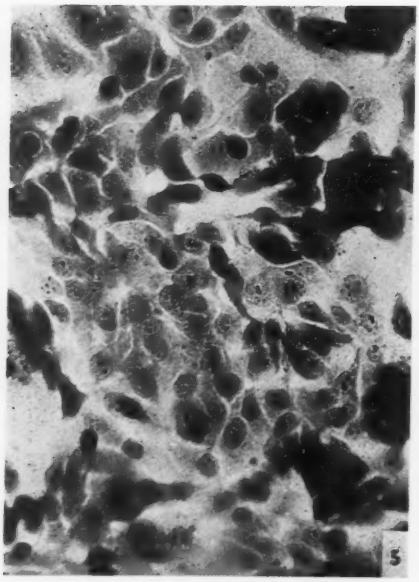

Fig. 5. 48 hrs control

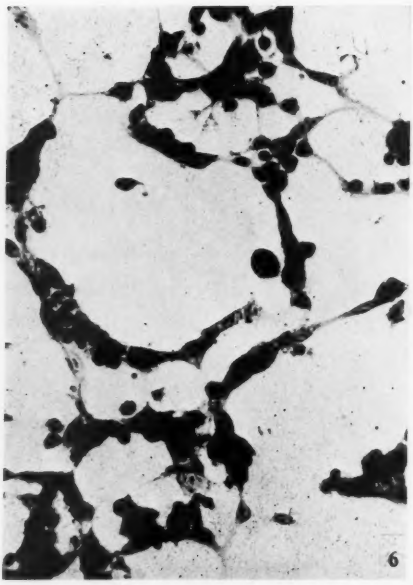

Fig. 6. 48 hrs. after the application of tetrahydrokinetin at the concentration of 1000 γ/ml

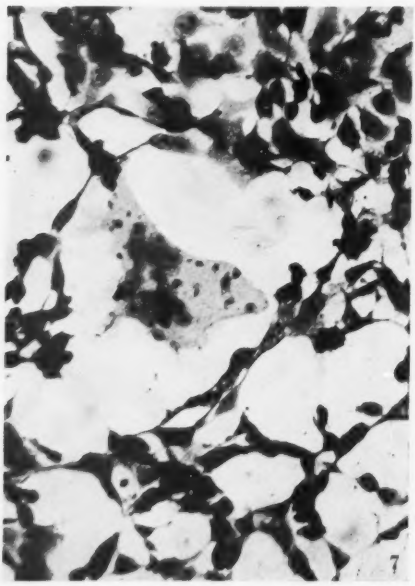

Fig. 7. 48 hrs. after the application of tetrahydrokinetin at the concentration of 500 γ/ml

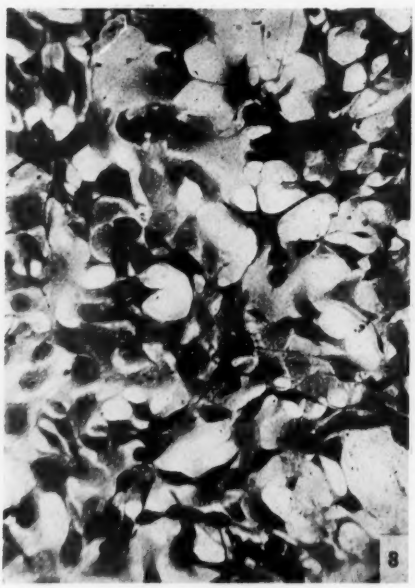

Fig. 8. 48 hrs. after the application of tetrahydrokinetin at the concentration of 125 γ/ml

[GANN, 49, 171~176; September, 1958]

STUDIES ON THE MECHANISM OF TOXOHORMONE FUNCTION. II

HIDEYUKI HOSHIZIMA

(Department of Biochemistry, Okayama University Medical School, Okayama)

Since the work of Nakahara and Fukuoka (1), it had been believed that normal tissues never yielded toxohormone by their method of preparation and that the partially purified toxohormone of Ono et al. (2) contained the same active principle as that of Nakahara. Ono et al., however, have recently demonstrated that normal rat serum treated by their method (2) (hot acetic acid-methanol extraction) gives a fraction which shows the similar catalase depressing activity, suggesting that normal serum constituents may acquire toxohormone-like character by this treatment (3).

In the previous paper of this series (4), it was reported that the toxohormone prepared by the method of Ono et al. (2) depressed liver ferritin iron paralleling with the depression of hepatic catalase activity after intraperitoneal injection into mice.

So it now becomes necessary to compare the effects of "toxohormones," prepared by these different methods, on the iron metabolism of mice.

Ascorbic acid has an intimate relation with the iron metabolism (5) (6) and most of the flavoproteins contain some metal ions (Fe, Cu, Mo) as the integral part of enzymes (7).

In this communication, the effects of these two toxohormone preparations on catalase, ferritin, ascorbic acid and riboflavin contents in the liver of mice were studied.

Materials and Methods

Toxohormone: Toxohormone was prepared by two different methods from surgically removed human gastric cancer tissues. One is the acetic acid-methanol extraction method of Ono et al. (2) and the other is the original method of Nakahara and Fukuoka (1), consisting of water extraction followed by alcohol precipitation.

Methods: (i) Experiments with toxohormone prepared by the method of Ono et al. 50mg of toxohormone fraction III was dissolved in the minimum amount of dilute hydrochloric acid and adjusted to pH 6 by alkali and was injected intraperitoneally into normal mice.

(ii) Experiments with toxohormone prepared by the original method of Naka-

hara and Fukuoka. 80mg of toxohormone was dissolved in the minimum amount of distilled water and injected intraperitoneally into normal mice. In both experiments liver catalase activity was assayed and expressed in k as described in the previous paper (4). Non-hemin iron of the liver was determined by the method of Yonekawa and Konno (8). Total ascorbic acid content of the liver was determined by the method of Roe et al. (9). Total flavin was determined by the fluorophotometric method of Yagi (10).

Results

The toxohormone was injected into mice intraperitoneally and twenty hours later liver catalase activity and nonhemin iron, ascorbic acid and riboflavin were determined.

Table 1. Total Ascorbic Acid Contents io Normal and Toxohormone (Ono's Preparation) Treated Mouse Liver (expressed by γ/g of wet liver)

Normal Mice		Toxohormone Treated Mice		
Exp. No.	Total Ascorbic Acid Contents	Exp. No.	Total Ascorbic Acid Contents	Catalase Activity k
12	365	10	326	2.5×10^{-3}
13	503	11	188	1.5×10^{-3}
15	460	13	290	2.2×10^{-3}
16	565	14	295	2.3×10^{-3}
17	570	15	376	2.8×10^{-3}
18	596			
20	476			

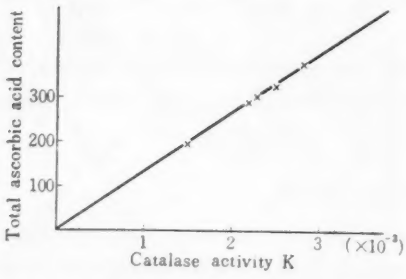

Fig 1. Parallelism Between Catalase Activity and Total Ascorbic Acid Contents in Toxohormone Treated Mouse Liver (Ono's preparation)

I. Experiments with toxohormone prepared by the method of Ono et al.

Table 1 shows the total ascorbic acid content in the liver of normal and toxohormone treated mice. Catalase activities (k) of each liver were also given in the Table 1 for reference.

Ascorbic acid was decreased in the toxohormone treated mice, and the depression of ascorbic acid was in parallel with the reduction of catalase activity of the same liver. This relation can be clearly seen in Fig. 1.

Table 2 shows the total riboflavin content in the liver of normal and toxohormone treated mice. No difference between normal and toxohormone treated mice was

Table 2. Total Flavin Contents of Normal and Toxohormone (Ono's Preparation) Treated Mouse Liver (expressed by γ/g of wet liver)

Normal Mice		Toxohormone Treated Mice		
Exp. No.	Total Flavin Contents	Exp. No.	Total Flavin Contents	Catalase Activity k
21	20.5	16	31.3	2.83×10^{-3}
22	19.9	17	20.2	2.3×10^{-3}
24	20.8	18	21.3	2.7×10^{-3}
25	22.8	19	20.4	2.6×10^{-3}
27	19.1	20	20.3	2.3×10^{-3}
		21	28.8	2.5×10^{-3}
		22	21.8	1.5×10^{-3}

observed.

II. Experiments with toxohormone prepared by the method of Nakahara and Fukuoka.

The liver catalase activities of normal and toxohormone treated mice are shown in Fig. 2.

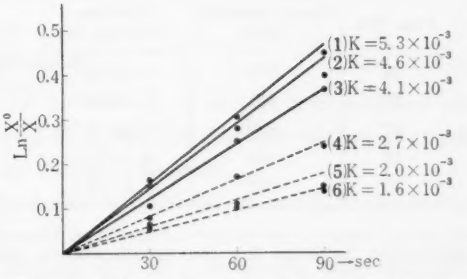

Fig 2. Liver Catalase of Normal and Toxohormone Treated Mice (Nakahara's Preparation)

Tables 3 and 4 show the non-hemin iron contents in the liver of normal and toxohormone treated mice. PI, PII, PIII, and SIII represent hemosiderin, ferritin, nucleoprotein bound iron and free iron respectively.

As compared with Table 3, only PII fraction (ferritin fraction) of the Table 4 showed a remarkable depression. But the depression of ferritin iron was not paralleled with the reduction of catalase activity. In this nature Nakahara's toxohormone preparation is different from that of Ono.

As shown in Tables 5 and 6, ascorbic acid content of mice liver was not affected by the administration of Nakahara's toxohormone. This is another point of

Table 3. Non-hemin Iron of Normal Mice (γ/g of wet weight)

Exp. No.	PI	PII	PIII	SIII	Catalase Activity (k)
10	142	132	83	28.5	5.0×10^{-3}
11	77	74	51	11	4.0×10^{-3}
13	41	48	29	3	5.0×10^{-3}
15	148	48	64	27	3.6×10^{-3}
16	150	148	76	23	4.0×10^{-3}
mean value	111.6	90	60.6		

Table 4. Non-hemin Iron of Toxohormone Treated Mice (Nakahara's Preparation) (γ/g of wet weight)

Exp. No.	PI	PII	PIII	SIII	Catalase Activity (k)
17	119	46	76	14	3.3×10^{-3}
18	102	26	68	8.6	2.0×10^{-3}
19	128	65	90	16	2.0×10^{-3}
21	47	19	23	8	3.0×10^{-3}
23	50	59	36	12	3.0×10^{-3}
24	87	35	36	23	3.3×10^{-3}
Mean value	80.5	41.6	54.8	13.6	

Table 5. Total Ascorbic Acid in Normal Mouse Liver (γ/g of wet weight)

Fxp. No.	Total Ascorbic Acid	Catalase Activity (k)
25	575	4.2×10^{-3}
26	575	5.7×10^{-3}
27	620	4.8×10^{-3}
28	558	4.3×10^{-3}
30	470	4.8×10^{-3}

Table 6. Total Ascorbic Acid in the Liver of Mice Treated with Toxohormone (Nakahara's Preparation) (γ/g of wetweight)

Exp. No.	Total Ascorbic Acid	Catalase Activity (k)
42	520	2.6×10^{-2}
43	475	2.7×10^{-2}
45	625	2.0×10^{-2}
46	558	2.5×10^{-2}

difference between the two toxohormone preparations.

Tables 7 and 8 show the total riboflavin content of normal and toxohormone treated mice liver. No difference was observed.

Table 7. Total Flavin Contents of Normal Mouse Liver (expressed by γ/g of wet liver)

Exp. No.	Total Flavin Content	Catalase Activity (k)
37	33.5	4.6×10^{-3}
38	35.2	5.0×10^{-3}
39	28.5	3.6×10^{-3}
40	23.4	3.3×10^{-3}
41	27.8	5.3×10^{-3}

Table 8. Total Flavin Contents of Toxohormone Treated Mouse Liver (Nakahara's Preparation) (expressed by γ/g of wet liver)

Exp. No.	Total Flavin Contents	Catalase Activity (k)
31	25.3	2.0×10^{-3}
32	28.1	2.3×10^{-3}
34	31.0	2.1×10^{-3}
35	30.3	3.0×10^{-3}
36	30.0	2.7×10^{-3}

Discussion

Toxohormone prepared by Ono's method of acetic acid-methanol extraction, induced parallel depression of catalase activity, ferritin iron and ascorbic acid of the mice liver.

Toxohormone prepared by the alcohol precipitation method of Nakahara and

Fukuoka also lowered catalase activity and ferritin iron of mice liver but not ascorbic acid. Furthermore, depression of catalase by Nakahara's toxohormone was not in papallel with that of ferritin iron.

Although more definitive study has to be carried out relative to the identity of the active principle included in the two preparations of toxohormone; one of the reasons of the different effects of two toxohormone preparations on the iron metabolism of mouse liver may be attributed to some contaminating substances in the toxohormone preparations.

The fact, that the reduction of catalase activity paralleled with the depression of ferritin iron and ascorbic acid of mouse liver after administration of Ono's preparation of toxohormone is in itself worthy of attention in relation to a report which indicates that ascorbic acid is capable of mediating the in vitro uptake of ferric iron by apoferritin (6).

Summary

Toxohormone prepared by the method of Ono et al. (acetic acid-methanol extraction) induced a parallel depression of total ascorbic acid and catalase of mouse liver, and showed no effect on the level of liver riboflavin.

Toxohormone prepared by the original method of Nakahara and Fukuoka (water extraction and alcohol precipitation) showed no effect on the level of liver ascorbic acid and riboflavin, but it depressed catalase activity and ferritin iron, although the extent of their depression was not in parallel.

The author wishes to thank Prof. S. Mizuhara and Mr. K. Okuda for their helpful suggestions. This investigation was aided in part by a research grant from the Ministry of Education.

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要　旨

Toxohormone の作用機転に関する研究

星　島　秀　行　(岡山大学医学部生化学教室)

小野等の醋酸メタノール抽出法により調製された Toxohormone を廿日鼠に注射し，肝臓の鉄代謝に関係ある事が知られている物質 (Vit. C, Vit. B_2, Catalase) を測定したところ，Vit. C は Toxohormone 注射により Catalase の減少と比例して減少する事がわかった。然し，Vit. B_2 の増減は見られなかった。

中原，福岡の原法 (水抽出アルコール沈澱) により調製された Toxohormone を用い，同様な実験を行ったところ，肝臓の Ferritin 鉄は減少する事がわかったが Vit. C, Vit. B_2 は対照との間に差が認められなかった．然も Ferritin 鉄の減少は Catalase の減少と比例しなかった。

(文部省科学研究費による)

[GANN, 49, 177~191; September, 1958]

INDUCTIVE EFFECT OF RAT ASCITES HEPATOMA ON THE AMPHIBIAN COMPETENT ECTODERM[1)]

(Plate XXI)

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INTRODUCTION

Since Spemann's discovery (1918) of the organizer in the amphibian embryo, many embryologists have attacked the subject from various angles. Each region of the organizer shows regionalities with respect to its capacity to induce differentiation of tissues by acting on a presumptive ectoderm (Spemann, 1931; Holtfreter, 1938: Hall, 1937: Kawakami, 1943, '48, '52). As the regionalities seem to have a principal relation to regional differentiations in animal development, a great deal of study has been done in connection with this problem.

Although the chemical relations between the inductive agent of heterogenetic tissues from mammals and that of the amphibian organizer have not been demonstrated, studies of the specific inductive activities of the former may contribute very helpful and constructive suggestions for the analysis of the regionalities of the organizer. In fact, investigations on the heterogenetic inductors such as liver, kidney and bone-marrow have furnished many valuable data (Holtfreter, 1933; Chuang, 1940; Toivonen, 1940; etc.).

In general, mammalian liver tissue produces archencephalic structures, kidney tissue induces deuterencephalo-spinocaudal structures and bone-marrow has a strong mesoderm-inducing capacity (Toivonen, 1940, '53; Yamada, 1950; Kawakami & Mifune, 1956). In contrast to the unstable nature of the mesoderm-inducing agent of the organizer, the agent of the adult tissues is comparatively stable under various treatments. This characteristic of the latter makes it easier to extract the agents from tissues and to analyze the difference and relation between the archencephalon-inducing mechanism and that inducing caudal structures or mesoderm. Even specific inductive capacities of the adult tissues, however, are modified by some treatments of the donor animal such as bud feeding (Toivonen, 1951; Engländer, Johnen and Vahs, 1953), x-irradiation (Kawakami & Mifune

1) This work was supported by the Scientific Research Funds from the Ministry of Education and the Asahi Shinbun Sha.

1957) and injection of nitrogen mustard-N-oxide (Mifune, 1957). In all these cases, the archencephalic or neural inductive tendency increases in the inductions produced by bone marrow which normally induces caudal or mesodermal structures. This seems to furnish a clue to the chemically intimate relation between the archencephalon- and mesoderm-inducing agents (Yamada & Takata, 1955 a; Kawakami & Mifune '57). The author is confident that studies on the alteration of inductive capacity of some tissues by means of various physiological conditions will give suggestions concerning not only the nature of the inductive agents, but also the physiological or biochemical changes in these tissues.

The inductive effect of malignant tissues has been brought to the attention of the embryologist principally from two standpoints. Giving thought to the anaerobic glycolysis of malignent tissues, which is as active as that of the orgnezer, Woerdemann (1933) tested their inductive effects. Although he demonstrated the neural inductive effect of Walker rat carcinoma and Rous chicken sarcoma, no specific action was noticed in the inductions by these tissues.

On the other hand, Waddington and his school (1934-1935) obtained neural induction by using steroid substances extracted from newt embryos and mammalian liver. Waddington (1938) then extended his experiment to a study of the effect of synthetic polycyclic hydrocarbons, and reported the inductive action of carcinogens such as methyl cholanthrene, styryl blue, and 3: 4-benzpyrene. In these experiments, however, the cultivation period of the operated larva was too short to identify the nature of the inductions, and thus, to draw a distinct conclusion on the inductive effect of malignant tissues and carcinogenic agents. Moreover, the inductive action of carcinogenic hydrocarbons may be explained as an unspecific toxic or sublethal cytolyzing effect on the reactor system which result in neural induction without respect to the kind of agent.

The author and Mifune (1957) have made a preliminary report concerning the inductive faculty of human penis cancer. The effect was almost absolutely mesodermal, as opposed to that found in the control experiment, in which the healthy part of epithelium was used as inductor and the neural inductions were of frequent occurrence in addition to the mesodermal ones. According to the histological observation, however, in almost all the cases of the control experiment, it was found that simultaneous grafting of connective tissues had been done together with the epithelium, because the healthy epithelium is very thin and its separation from the underlying connective tissue is very difficult. Therefore, a reliable conclusion could not be drawn from the data. To reconfirm the special inductive capacity of the penis cancer, another experiment was planned, but during the season of experiment in 1957 no case of the cancer visited the

Hospital of the Medical Department of Kyushu University and a repetition of the experiment could not be done.

If some special nature can be found in the inductive capacity of malignant tissues, we may be able not only to approach the pathological characteristics of the tissues from the embryological angle, but to find a means of studying the nature of the inductive agents from the pathological angle.

For these studies hepatoma seems to be one of the most suitable materials, since the normal liver has a comparatively simple, homogeneous histological structure and strong archencephalic or archencephalo-deuterencephalic inductive capacity. Moreover, when liver, ascites hepatoma or nodules of the cancer of rat is used as inductor material, enough tissue is available to fractionate the cellular components. The present experiment is concerned with the inductive effect of the ascites hepatoma.

Materials and Methods

The presumptive ectodem of ***Triturus pyrrhogaster*** gastrulae, which were collected in ponds on the campus of Kyushu University, was used exclusively as the reactor material.

As the inductor tissues, nodules of two kinds of ascites hepatoma obtained from the Sasaki Laboratory in Tokyo was used. The serial transplantation of the hepatoma was done every 10 th day in our laboratory, using hybrid male rats weighing about 200 g.

These ascites hepatomas, which are designated as AH 7974 and AH 130, are easy to transplant and the transplantation rate is close to 100 per cent. There is a marked difference in sensitivity to nitrogen mustard-N-oxide between these two hepatomas; AH 7974 is very resistant and AH 130 is highly sensitive to the chemicals (T. Yoshida et al., 1956). The formation of nodules takes place in nearly all animals in which the transplantation has been done successfully.

The nodule of ascites hepatoma was removed 11 or 12 days after transplantation and preserved in 70 per cent ethanol at 2°C until the grafting was done, but in 4 series fresh cancer tissue was used. In this experiment, the cancer tissue pieces to be explanted were taken from the inner part of the nodules to avoid simultaneous transplantation of peritoneal tissue. The *Triturus* eggs were disinfected with 0.06 per cent chloramine and all procedures of operation and cultivation were done under sterile conditions. A small piece of the cancer tissue, which was washed with Holtfreter's salt solution for about one hour, was sandwiched in the competent ectoderm. After cultivating the sandwich in Holtfreter's solution for 10 or 11 days, it was fixed with Bouin's solution.

As control experiments, two series were performed; in the first, the develop-

mental potency of the competent ectoderm was tested by culturing the tissue alone; and in the second, the inductive effect of normal rat liver tissue was tested with the same method as the experimental series.

RESULTS

I. **Control experiments:**

a) **Self-differentiation of the competent ectoderm.** The piece of competent ectoderm to serve as the reacting material was cut from the presumptive epidermal and neural region, care being taken not to carry the adjacent chorda-mesoderm and other marginal zone materials with the piece. As has been reported in various amphibian eggs, such a piece did not undergo special differentiation, forming only irregular epidermis. In all 61 cases in the present experiment also, there was no differentiation and it may be said that pure presumptive ectordermal material had been taken out.

b) **Inductive effect of the normal rat liver tissue.** Except for the data of Chuang (1940) in *Triturus*, the liver tissue generally shows a strong archencephalic or archencephalo-deuterencephalic inductive effect. Mouse liver tissue used as inductor gave archencephalic induction in the experiment of the author and Mifune (1956), and Hama (1944) showed the same effect in rat liver tissue. In this series also, archencephalic induction was naturally anticipated and the results fulfilled this expectation. That is, 16 explants out of 23 produced solely neural inductions and 10 of them could be clearly identified as archencephalon (Fig. 1), with the characteristic structures and sensory accessaries. The remaining 6 inductions were large, poorly differentiated neural cell masses. The other 7 cases were negative and produced no induction.

In 2 of these negative cases mesodermal cells were observed, but amount of such tissue was too small to attribute the differentiation to the inductive effect of the liver tissue. Therefore, in the present work also the archencephalon-inducing action of the normal liver is confirmed. Althouth the liver tissue consists of many kinds of cells, since there is an absolute majority of hepatic cell among them, it may be said that the specific inductive capacity of the liver tissue is led by the hepatic cells.

II. **Experimental series:**

There are some morphological differences between AH 7974 and AH 130, but one of the most notable differences is in the sensitivity to nitrogen mustard-N-oxide, as mentioned above (Yoshida et al., 1956). Considering the modification of the mesoderm-inducing action of mouse bone-marrow by the chemicals, the author paid attention to the difference in the inductive actions of the two hepatomas. Thus, to test the stability of the inductive agents in each hepatoma,

the malignant tissue was grafted after preservation in cold 70 per cent ethanol for 1, 24, 72, 120 hrs. and 8 days

1) **Inductive effect of nodules of AH 7974:**

a) **Inductions by the fresh tissue.** The nodule material was taken from 2 donor animals, and pieces taken from the inner part of the nodule were grafted in the fresh condition. In these two groups of experiment, 58 and 31 usable cases were obtained, respectively. Histological studies of the first series failed to detect the grafted cancer tissue in 11 cases. When the tissue fixed with ethanol had been used as inductor, the grafts were preserved better as described below, so it may be presumed that the grafts in these 11 cases were absorbed during cultivation. The remaining 47 cases had comparatively well preserved grafts, but only 2 cases realized neural induction, except for 6 cases in which poor mesodermal tissue differentiation took place.

One of the two neural inductions formed a comparatively large neural mass and was obviously a part of the brain, but its further identity could not be established. The other was surrounded by mesemchyme cells and accompanied by two ear vesicles, indicating a deuterencephalic induction.

In the latter series, the grafted cancer piece was preserved rather well in all 31 cases, in contrast with the previous series. Neural and solely mesodermal inductions were observed in 4 cases each, and the remaining 23 cases were negative. In 2 cases, neural inductions were produced without any mesodermal cells and seemed to be archencephalic. One was an unspecifiable bain and the other, surrounded by mesenchyme cells, showed a deuterencephalic nature. In 4 cases accompanied by mesodermal differentiation, mesenchyme cells, cavities lined with thin epithelium and poorly differentiated muscle tissues were found. Comparing these structures with the mesodermal cells which were observed in the control series in which the normal liver tissue was used as inductor, the former were vastly superior in amount and differentiation of mesodermal tissues. Thus, these mesodermal structures in this series, or at least some of them, may be attributed to the inductive effect of the cancer tissue. These mesodermal tissues, however, seem to be evoked by a weak mesoderm inducting effect, while the induction of notochord, muscle and pronephros is realized by the stronger effect. It may therefore be concluded that the fresh nodule of ascites hepatoma has only a weak inductive action.

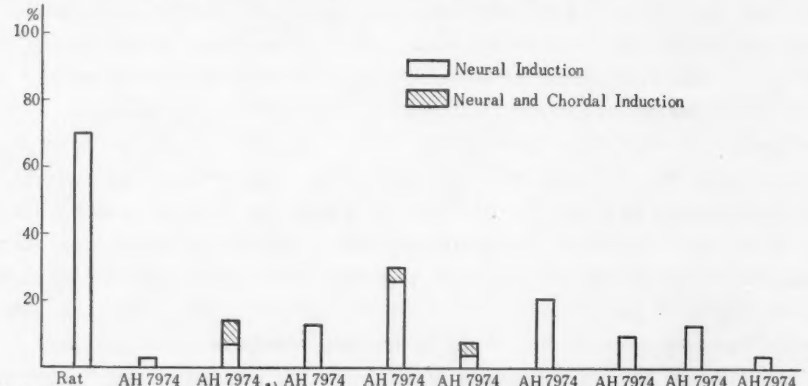
b) **Inductions by ethanol-fixed tissue.** Using the nodules of AH 7974 fixed by ethanol for 2 hour, two series of experiments were performed. The grafted cancer tissues were found in a well-preserved condition. The neural induction was produced in 7 and 6 cases out sf 23 and 41, respectively. The neural frequencies in these series were comparatively high, and moreover, the differentia-

tion of these inductions was of rather good quality, as follows.

In the first group, 1 deuterencephalon, 2 deuterencephalo-spinal induction and 1 questionable archencephalon; and in the second, 2 archencephalons and 1 spinal cord were induced. The other neural inductions were 1 neural fragment and 2 neural cell masses in each.

Mesodermal induction was of comparatively rare occurrence, but 3 notochord inductions (Fig. 2) in the first series and 1 in the second were noticed. All these notochords occurred together with neural inductions, which were deuterencephalon accompanied by ear vesicle and spinal cord. On histological examination of these cases, no other tissues other than cancer cells were observed. This indicates that the notochord inductions were realized by the effect of the hepatic cancer cells.

In the other experimental group, the explantations were done after keeping the alcohol-fixed cancer tissue in a refrigerator for 1, 3 and 5 days. In these series, 26, 41 and 14 usable cases were obtained, respectively. In all these series, neural inductions were rare occurrences, and moreover, no notable differentiation, but only fragmental neural structures, were evoked. One of 2 neural fragments obtained in the one-day series was accompanied by notochord induction. Mesotherial and mesenchyme differentiations in the one-day series were frequent, but in the 3- and 5-day series they were extremely rare.



Nerve	16	2	6	4	7	2	7	4	2	1
Notochord	0	0	3	0	1	1	0	0	0	0
Mesoderm	0	6	5	4	3	11	9	1	1	9
Negative	7	50	30	23	13	13	11	11	36	13
Total	23	58	41	31	23	26	27	14	14	23

1) Fixed by ethanol for 2 hrs. 2) Grafts were taken from the same donor.

Chart 1. Neural and notochordal inductions by rat normal liver and nodus of ascites hepatoma AH 7974.

The nodule which was taken from another animal was used 1 and 8 days after fixation. Although the first series produced a comparatively high percentage of neural induction; i. e., 16 per cent, in the 8-day series, the induction was again of a low quality, as in the previous series, in which cancer tissues preserved for 3 and 6 days were used as inductor. There neural inductions were fragments except one archencephalic one (Fig. 3) and no notable mesodermal induction took place in these two series (Chart 1).

2) **Inctive effect of nodules of AH 130.**

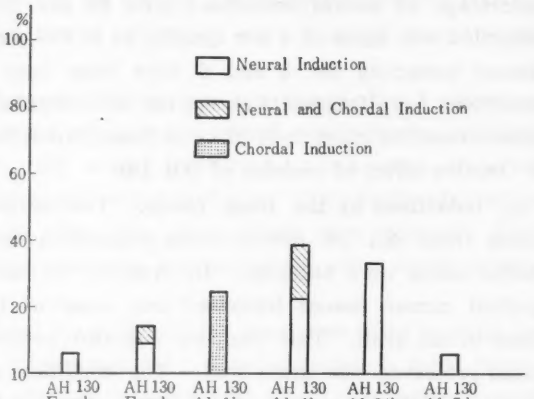
a) **Inductions by the fresh tissue.** Two series of experiments were made, using fresh AH 130 nodule tissue originating from 2 animals, and 31 and 22 usable cases were obtained. In most of the cases of the former series, the grafted cancer tissues histolized and some of them left behind only a slight trace of the graft. This tendency was also noticed in the latter series, but 7 cases preserved the graft well. The condition of the grafted tissue, however, had no relation to whether induction occurred or not. That is, even in some cases in which the graft was well preserved, no induction was evoked, and 3 neural tissues and a notochord were induced in explants in which the grafted cancer tissue was not detected in the explant. As the explants from which the graft had became separated were eliminated 3 days after operation, these inductions which occurred in the explants in which the graft coult not be found may have been evoked by the grafted cancer tissue before it was absorbed.

Neural inductions were obtained in 2 cases in each series. These tissues were poorly differentiated. One was spinal cord and the other 3 were neural fragments. A notochord induction was independent of the neural induction.

b) **Inductions by ethanol-fixed tissue.** The tissue of nodules of AH 130 was preserved in 70 per cent ethanol in the refrigerator for 1 hr., 24 hrs. and 5 days before explantation was performed. Comparatively frequent notochord induction (Fig. 4) was characteristic of inductions by the AH 130 cancer tissue fixed for 1 hour. In two groups of this series 20 and 12 available cases were obtained, and 3 notochord inductions were evoked in each. In the first group neural induction was also frequent; i. e., 7 out of 20 cases produced neural inductions. These consisted of one archencephalon (Fig. 5), one deuterencephalon, one unidentifiable brain, 3 neural fragments and one spinal cord. Three of these neural inductions, i. e., the deuterencephalic brain, spinal cord and one neural fragment, were induced together with notochord. One of the neural fragments was very small and looked like ear vesicle, and was surrounded by a compact cell mass. The nature of the cell mass could not be identified, but the component cells looked like mesodermal cells. In this group no neural induction could be found.

Thus, the inductions of these two group showed a deuterencephalo-spinocaudal and mesodermal tendency, except for one archencephalic induction.

However, the cancer tissue which was fixed for 24 hrs. evoked solely neural induction in 6 of 18 usable cases. These were: 2 archencephalic, I deuterencephalic and 3 neural fragments. Such a tendency for neural induction was also noticed in the series in which cancer tissue fixed for 5 days was used as inductor, but the induction frequency was low (6%) as in the previous series in which AH 7974 tissue fixed for a longer period was grafted. The induction was only one unidentifiable brain out of 16 usable cases (Chart 2).



Graft	AH 130 Fresh	AH 130 Fresh	AH 130 Al.1hr.	AH 130 Al.1hr.	AH 130 Al.24hrs.	AH 130 Al.5day
Nerve	2	2	0	7	6	1
Notochord	0	1	3	3	0	0
Mesoderm	3	6	2	2	0	1
Negative	21	13	7	11	12	14
Total	31	22	12	20	18	16

Chart 2. Neural and notochordal inductions by nodus of ascites hepatoma AH 130.

Discussion

Judging from the fact that the induction rate of both fresh samples of ascites hepatoma nodules is notably low, one may be able to conclude that these malignant tissues almost lack inductive capacity, at least in the fresh condition. Nearly the same result was obtained by Saxén and Toivonen (1956) using as inductor rat leucemi bone-marraw tissue fixed by ethanol. According to their experiment, the sarcoma tissue absolutely lacks in inductive action. In the present work, however, althouth the frequency of induction was markedly low compared with that of the normal rat liver tissue, the cancer tissue fixed by ethanol produced rather frequent inductions of neural and mesodermal tissues, including notochord. The tendency was especially marked in the series in which the cancer tissue fixed by ethanol for a short time was used as inductor. As mentioned in the previous section, peritoneal tissue was not noticed in the graft even in the cases in which notochord induction took place, so it may be said that these inductions principally depend upon the inductive action of the hepatoma cell itself. Considering the unstable nature of the mesoderm-inducing

agent with respect to nitrogen-mustard-N-oxide (Mifune, 1957), the author expected an earlier loss of mesodermal inductive action in AH 130 than in AH 7974, since the former hepatoma is more sensitive to the chemical than the latter. So far as the present experiments are concerned, however, no noticeable difference in inductive effect could be found between them.

One may infer from the scarcity of inductions by the fresh ascites hepatoma tissue that the comparatively high frequency of inductions by the tissue fixed in ethanol for a short period may be contributable to an agent newly produced by the ethanol treatment. Whatever the treatment is, however, the appearance of inductive capacity in non-effective tissue on its being killed is confined to neural induction. For example, noninductive presumptive ectoderm produces, on being killed no inductive effect other than neural. Therefore, it may be judged that at least the rather strong mesodermal inductive action of the fixed nodule tissue is inherent.

Now, there is another result of a preliminary experiment (Kawakami & Mifune, unpublished) which must be noted here; i. e., the crude proteins extracted from the nodule of ascites hepatoma with NaCl solution evoke a high frequency of inductions of the same kind of tissues as those which are produced by the hepatoma tissue. That is, 18 out of 23 usable cases were positive in induction; deuterencephalo-spinocaudal and mesodermal inductions being found in 13 cases, and archencephlic and unspecifiable brains in 5. Notochord induction occurred in 3 cases. This fact demonstrates that the cancer cell has a sufficient amount of agent to perform induction. Therefore, the weak inductive action of the ascites hepatoma tissue may be attributable to some other cause than the lack of inducing agent. In spite of some objections (Holtfreter, 1955), the author beleives that direct, intimate contact between inductor and reacting material seems to be indispensable to realize induction. Thus, there may be a possibility that some pathological change in the cell membrane of the hepatic cells, which inhibits the effective contact between the inductor tissue and the reacting materials, takes place on their becoming neoplastic. If this is the case, the inductions produced by the cancer tissue might presumably be due to cytoplasm which leaked from cells when the tissue was cut into pieces.

One of the significant facts concerning the inductive capacities of the nodules of ascites hepatoma is that the hepatic cells seem to lose their original capacity for inducing solely archencephalon, and acquire a mesoderm-inducing capacity. As to the mesoderm-inducing capacity of the nodules of ascites hepatoma, it is nearly certain that a slight contamination of other tissues than the hepatoma cells, such as blood cells, blood vessel, peritoneum, etc., is not the cause of this effect, since, as mentioned, on histological observation no cells other than

hepatoma cells were detected in the grafted nodule tissue. The author, therefore, is firmly convinced that the inductive capacity has been partially modified by the tissues becoming neoplastic. However, as all doubts concerning the possible induction of mesodermal tissues by contaminating cells of the nodules other than hepatoma cells are not necessarily eliminated and this problem seems to be very important in connection with the inductive capacities of malignant tissues, this point must be made sure by using homogeneous hepatoma tissue or free cells of ascites hepatoma as inductor in a future experiment.

The author (1956) sets up the following hypothesis on embryonic morphogenesis. In this hypothesis two main morphogenetic agents are assumed. One is concerned with archencephalic development and the other with chordal or mesodermal differentiation. Each of them may be some kind of protein (cf. Lehmann, 1945; Kussi, 1951; Yamada & Takada, 1955; Kawakami & Yamana, 1956) and may be designated as Ma and Mm respectively. After they appear in an undifferentiated tissue, each of them may act so as to determine the course of differentiation of the tissue, and, on the other hand, they may act as inductive agents by operating on competent ectoderm from outside. As substances which originate from different tissues produce the same inductive effects, Ma and Mm must be names for substance group, each of which includes various substances which have the same inductive effects, and presumably their unique effects depend upon a special common structure of each group of proteins. Thus, the inductive agents of the prechordal material of the amphibian gastrula and the guinea-pig liver may belong to Ma, and those of the posterior-most part of the archenteron roof of the late gastrula and guinea-pig bone marrow, to Mm. In this hypothesis, the author assumes the following posibilities with respect to the differential induction of the mesodermal structures and axial neural tissues. The notochord and other mesodermal structures such as muscle, pronephros and mesenchyme cells may result from a difference in the strength of the inductive effect of Mm; for example, the pronephric tissue is realized by a weaker effect of Mm than that which causes notochordal-induction. On the other hand, the nature of the axial neural induction may be determined by the ratio of the two agents which coexist in the inductor. The latter supposition was partly supported by the experiment of Kawakami and Yamana (1958); that is, deuterencephalic and spinocaudal induction were obtained by using mixed proteins of guinea-pig liver and bone marrow.

As for the chemical relation between Ma and Mm, the author attaches importance to the following facts. One of these is that uninvaginated prechordal material of the *Triturus* early gastrula produces spinocaudal induction, but its proper archencephalic inductive effect makes its appearance after invagination

(Okada & Takaya, 1943). A second is that the mesodermal inductive effect of mouse bone marrow is modiffed to be neural inductive by totally x-irradiating it (Kawakami & Mifune, 1957) and by injecting nitrogen mustard-N-oxide (Mifune, 1957). Moreover, an alteration giving rise to the same tendency takes place in proteins from guinea-pig kidney and bone-marrow when they are used many days after extraction (Yamada et al., 1955; Mifune, unpublished). These facts seem to show that the Ma originates from Mm through some chemical change.

In the author's hypothesis, another important supposition is that amphibian competent tissue lacks in the morphogenetic factors but has their precursors; and when the competent tissue comes in contact with inductors, the precursors become active by being assimilated to each of these morphogenetic (i. e. inductive) factors. Now, if mixed inductive agents (Ma+Mm) act on the competent material, the two kinds of morphogenetic factors may be produced in the competent material in the same ratio as the inductive agents in the inductor. This supposition may be supported by homeogenetic induction in the amphibian embryo (O. Mangold, 1933; Walter, 1948; Hildegard, 1953). That is, the inductive capacities of the neural plate region do not become effective until the presumptive neural plate acquires the self-differentiating faculty, and after the time each region of the plate evokes the structure which is identical with the inductor itself operating upon competent ectoderm.

Under this hypothesis, the author attached importance in the present study to quantitative and qualitative modifications of the inductive capacity of the hepatic cells caused by their becoming neoplastic. Concerning the reduction of inductive capacity of the cancer tissue, the author, as mentioned above, attributes the cause to a pathological change of the surface of cancer cells, but the localization and the effectiveness of inductive agents in the hepatoma cell components must be compared with those of normal hepatic cells before an ultimate conclusion can be reached. If the alteration of inductive capacity (Ma $\rightarrow$ Mm) in hepatoma cells is indeed the case, the alteration may be considered to be concerned with the aberration of growth of tumor tissue. The author assumes that an interpretation other than that of Saxén and Toivonen (1956) must be placed on the relation between inductive faculty and growth of malignant tissue. They discussed the relation on the basis of their data, in which no inductive effect of sarcoma was shown. As these authors pointed out, it may be true that growth and differentiation are distinguishable as two separated events, which are regulated by different mechanisms. But their experimental data seem to be insufficient basis for their conclusion, since according to the present author's preliminary experiment, the positive inductive effect of tumor tissue seems to be evident, at least to the extent that nodules of

hepatoma ascites and the tumor cells seem to contain comparatively strong inductive agents. At present the author is inclined to supposed that there is an increase of a growth-promoting factor in tumor cells, which is presumably produced in connection with the alteration of the morphogenetic (inductive) factor, and that aberration of growth disturbs the regulatory effect or marphogenetic factors. Although the decisive conclusion cannot be made from the present data, the author assumes at least in some cases the probable coexistence of Mm and a growth promoting factor, without suggesting that these two factors are identical, taking the strong mesoderm-inducting capacity of chick embryo extract (Woellwarth, 1956; Tiedemann, 1957; Sasaki & Kawakami, unpublished) into account. Moreover, another probability must be considered in connection with the poor differentiating power of tumor tissue. In morphogenesis of tissue, not only the differentiation of cells but also a constructive affinity and interdependence among the component cells may be necessary. Although one has no experimental method to test these points, some abnormality may be predicted in such an aberrant tissue as tumor. And does it go too for to say that the lack of interdependence among tumor cells may be due to the same pathological change in the cell membrane as that which seems to be the cause of reduction of inductive effect of tumor tissue?

At any rate, concerning the inductive capacity of the tumor tissues many problems are left for solution, and the author believes that much importance must be attached to these problems.

Summary

1. The inductive effects of nodules of the ascites hepatoma of the rat were tested, using the presumptive neuro-epidermis of *Triturus* gastrula as reactor.

2. Two kinds of the ascites hepatoma, AH 7974 and AH 130, which have different morphological and pathological characteristies, were used.

3. The experiments were done by the usual sandwich explantation method. A small piece of fresh or ethanol-fixed cancer tissue was sandwiched between pieces of presumptive ectoderm.

4. In the control series the normal rat liver tissue induced solely neural tissue, especially archencephalic structures.

5. The inductive capacity of fresh tissue of the nodule of each cancer was notably weak and the rate of induction was not over 15 per cent, against that of the normal hepatic tissue of 70 per cent.

6. The cancer tissue fixed by ethanol for short periods (from 1 to 24 hrs.) produced inductions in rather high percentage, but protracted ethanol treatment (from 3 to 8 days) reduced induction frequency to percentages as low as those

in the fresh cancer tissue series. There were no detectable differences in the inductive action between AH 7974 and AH 130.

7. The weak inductive effect of the cancer tisssue seems to depend upon a pathological change of the hepatic cell surface, since in a preliminary experiment the crude proteins extracted from nodules of ascites hepatoma showed a strong inductive effect.

8. Some inductions by the fresh cancer tissue or cancer tissue fixed for short periods in ethanol showed spino-caudal characteristics and were occasionally accompanied by notochord differentiation. The mesoderm-inducing tendency seems to be due to a modification of the inductive capacity of the hepatic cells caused by their becoming neoplatic.

Grateful acknowledgment is made to Dr. W. Nakahara, of Cancer Institute (Japanese Foundation for Cancer Research), for his kind encouragement. The author also wishes to express his gratitude to Mr. S. Mifune and Miss K. Eshima, of Kyushu University, for their technical assistance.

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Explantation of Figures. Plate XXI

1. Archencephalic induction by rat fresh liver tissue. N: nose, Fb: fore-brain. X 100.
2. Spinocaudal induction by tissue of nodule of AH 7974 fixed with ethanol for 2 hrs. Not: notochord, Sp: spinal cord. X 130.
3. Archencephalic induction by tissue of nodule of AH 7974 fixed with ethanol for 8 days. Fb: fore-brain. X 130.
4. Mesodermal induction by tissue of nodule of AH 130 fixed with ethanol for 1 hr. No neural tissue is noticed. Not: notochord, Mes: mosodermal cells. X 130.
5. Archencephalic induction by tissue of nodule of AH 130. Fb: fore-brain. X 130.
6. Showing irregularly differentiated epidermis in negative case. Graft from nodule of AH 7974 was used. G: graft. X 130.

要　旨

腹水肝癌の誘導効果

川上　泉（九州大学理学部生物）

シロネズミの正常肝組織をイモリの嚢胚の予定外胚葉に作用させると原脳的構造を誘導する。この実験では，同種の腹水肝癌結節 (AH 7974, AH 130) を誘導原に用い，癌性化することにより誘導効果が如何に変化するかを見た。 1) 正常肝組織は原脳的誘導を含む 70% の神経性の誘導を起した。両肝癌結節では誘導率は極めて低い。(3～39%) 両種肝癌に差異は認められなかった。 2) 予備的実験で，0.14 M NaCl で結節から抽出した粗核蛋白質に強い誘導能が見られたところから，肝癌細胞は誘導能を内包するが，細胞膜にその誘導作用を防げる如き病理変化があるように思われる。 3) これらの肝癌結節で，しばしば，背索の誘導が見られた。正常肝細胞の原脳誘導性が癌性化により中胚葉誘導の効果を帯びるようになると考えられる。

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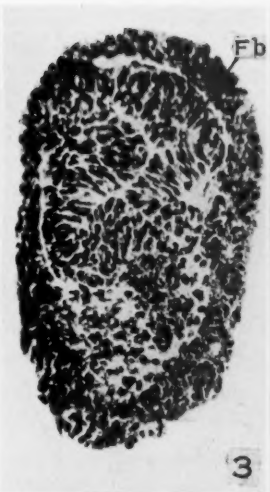
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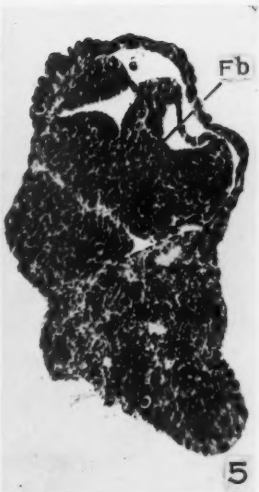
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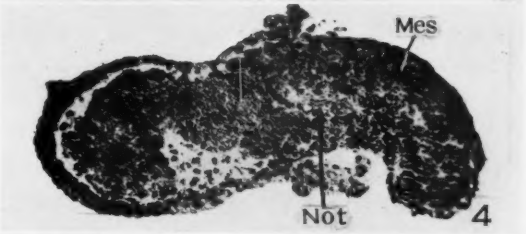
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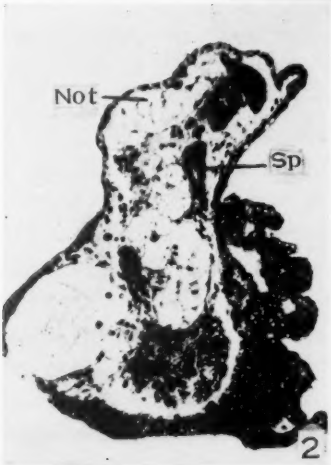
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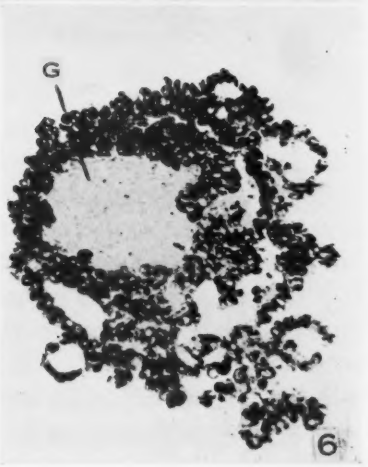
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[GANN, 49, 193~198; September, 1958]

INFLUENCE OF LOADING KUPFFER CELLS ON THE DAB-DESTROYING ABILITY OF RAT LIVER SLICES

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(Director: Prof. Hisamasa Sato)

Sato (1) and Kensler (2) reported sometime ago that p-dimethylaminoazobenzene (DAB) was destroyed by surviving rodent liver slices. Furthermore, Sato (3) has reported that liver slices from rats, mice, and rabbits showed different abilities of destroying DAB, the ability being highest in rats and lowest in rabbits. After that, Kawai (4) reported that the above ability was increased when riboflavin was added.

Kinoshita (5-6) suggested that the reticulo-endothelial system might be involved in the azo dye carcinogenesis. More recent report by Baillif (7) revealed that there exists a close relationship between the reticulo-endothelial system and the growth of the tumor. However, it has not been settled whether or not Kupffer cells participate in the metabolism of DAB.

This study was undertaken to evaluate the influence of loading Kupffer cells on the DAB-destroying ability of rat liver slices. The loading was effected by India ink, trypan blue, congo red, or carmine. In this investigation, the author has intended to study the relationship between the DAB-destroying ability and the degree of loading in hepatic reticuloendothelial cells (Kupffer cells).

Materials and Methods

A total of 132 hybrid albino rats, weighing 100 to 200g was used. They were divided into five groups including a control group which received 0.9 per cent NaCl solution. Group 2 received 3 per cent solution of India ink in saline. Group 3 received 1 per cent solution of trypan blue in saline. To group 4 was given 1 per cent solution of congo red in saline. To group 5 was given the mixture of 3 per cent solution of India ink and 0.08 per cent solution of carmine in saline. Each rat was given an injection of 10cc per kg of body weight of each solution by the intraperitoneal route. After certain hours (3, 6, 9, 12, 18, 24, or 48 hours), they were sacrificed by decapitation. The livers were quickly exised, and transfered to ice-cold Krebs-Ringer phosphate solution, pH 7.4. After that, they were cut into slices, and 200mg of them were suspended in 20cc of Krebs-Ringer phosphate solution saturated with DAB and were shaken in water bath at 37.5°C. while be-

ing supplied with oxygen for 30 minutes. Immediately afterwards the solution was boiled, to which was added 2cc of 25 per cent alcohol-hydrochloric acid. The light-absorption data were obtained with a Beckman-type spectrophotometer with a slit-width of approximately 0.03mm, and a 10mm wide corex cell. The amount dissolved was determined spectrophotometrically.

At the same time, a specimen of the liver in each rat was fixed in 10 per cent Formalin, Susa solution, or absolute alcohol. Each specimen was embedded in paraffin blocks and stained with Hematoxylin-Eosin, van Gieson, PAS, and Safranin, for the study of the relationship between the DAB-destroying ability of rat liver slices loaded with carbon granules and the degree of its storage in Kupffer cells. The average number of Kupffer cells laden with carbon granules was caluclated within a sweep of microscope (40×10) surrounding a *V. centralis.*

Results

The DAB-destroying ability of the surviving rat liver slices is shown in Table 1, Fig. 1, and Fig. 2. In group 2 loaded with carbon granules, the ability was

Table 1. DAB-destroying ability of rat liver slices. Average values (r/10cc) of the destruction of DAB in 30 minutes.

Groups \ Hours	3	6	9	12	18	24	48
Control (Group 1)	3.1 (2.7—3.4)	3.1 (2.5—3.4)	3.2 (2.8—3.5)	3.1 (2.8—3.5)	3.1 (2.9—3.5)	3.2 (2.6—3.5)	3.2 (2.7—3.3)
Group 2 (India ink)	2.2 (1.7—2.8)	2.1 (1.8—2.4)	2.6 (2.3—2.9)	2.6 (2.0—2.9)	3.2 (2.5—4.0)	2.8 (2.3—3.3)	2.4 (2.1—2.7)
Group 3 (mixture)	3.1 (3.0—3.3)	2.8 (2.5—3.6)	3.8 (3.4—4.4)	3.8 (3.3—4.0)	2.0 (1.8—2.5)	1.9 (1.8—2.2)	2.2 (1.5—2.7)
Group 4 (Trypan blue)	3.2 (2.8—3.8)	2.4 (2.0—2.9)	2.5 (2.2—2.8)	2.4 (1.8—2.8)	—	3.0 (2.8—3.4)	2.2 (1.5—2.8)
Group 5 (Congo red)	2.7 (2.5—3.1)	2.8 (2.5—3.3)	2.5 (2.1—2.8)	2.4 (2.0—2.8)	—	2.4 (1.9—2.8)	1.8 (1.6—2.0)

Each figure shows the mean value in 3 to 5 rats.

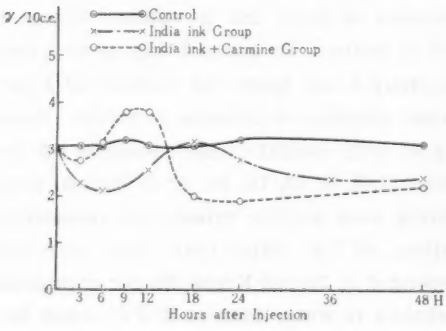

Fig. 1. Comparison curves derived from values of DAB destroyed by rat liver slices in India ink and mixture group. The 200mg of rat liver slices were suspended in 20cc of Krebs-Ringer phosphate solution saturated with DAB and were shaken in water bath at 37.5°C, while suppling oxygen for 30 minutes. The amount dissolved was determined spectrophotometrically. Each point shows the mean value in 3 to 5 rats.

lowest 6 hours after injection, and then after approaching the control level in 18 hours and roughly maintaining the level up to 24 hours, it gradually subsided. While in group 5 with the mixture of carmine and India ink, the destroying ability was highest 9 to 12 hours after injection, and lowest after 18 hours, but it was gradually brought uphill from this point. In group 3 with trypan blue, the ability was slightly accelerated 3 hours after injection, and was kept week during 6 to 12 hours, but gradually rising again, it approached the control level after 24 hours. Thereafter, however, the ability was similarly weakend as in the case of carbon granules. In group 4 with congo red, DAB-destroying ability was gradually weakend 3 hours after injection. Furthermore, the abillity in the congo red group was continued in the depressed state to the end of the experiment. However, in the control group which had received 0.9 per cent NaCl solution, the DAB-destroying ability was almost invariable throught.

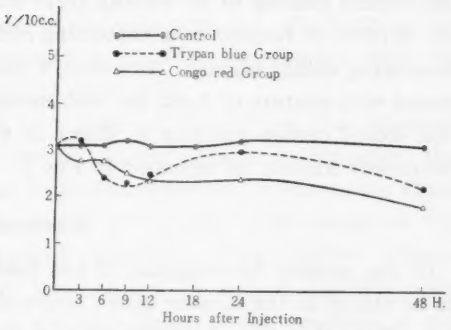

Fig. 2. Comparison curves derived from values of DAB destroyed by rat liver slices in trypan blue and congo red group. The materials and methods are the same as in Fig. 1. Each point shows the average value in 3 to 5 rats.

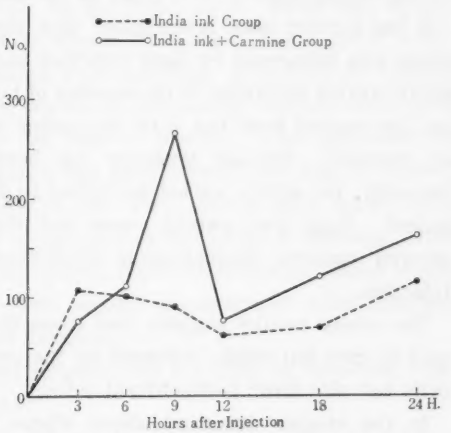

Fig. 3. Number of carbon granules stored in Kupffer cells. The average number of laden Kupffer cells storing carbon granules was calculated within a field of microscope (X 400) surrounding a *V. centralis.*

Microscopically, the carbon granules was already recognized in Kupffer cells 3 hours after an intra-peritoneal injection. The other dyes were clearly recognized in Kupffer cells 12 hours after injection. The Kupffer cells varied in degree of hypertrophy according to the kinds of dyes 12 hours after injection, but was prominent in congo red group. The carbon granules appearing in cytoplasm of the Kupffer cells had various shapes such as those of rads, or threads. Some broken Kupffer cells were recognized in a hepatic lobule 48 hours after injection. In the relationship between the DAB-destroying ability of rat liver slices loaded with carbon granules and the

histological grading of its loading there was to be seen a tendency that the larger the number of Kupffer cells containing carbon granules was, the lower the DAB-destroying ability became. However, 9 hours after injection, the rat liver slices loaded with mixture of India ink and carmine had larger number of Kupffer cells that stored carbon granules as shown in Fig. 3, but DAB-destroying ability was conversely highest as indicated in Fig. 1.

Discussion

In the present investigation, it has been observed that carbon granules began to be stored in the Kupffer cells 3 hours after the intraperitoneal injection of India ink. The other dyes, however, were recognized in the Kupffer cells by degrees 12 hours after their injections. The number of Kupffer cells storing carbon granules decreased in number after 24 hours. But some broken Kupffer cells remained predominantly in the centrol zone of a hepatic lobule after 48 hours. These results agreed with those in Ishibashi's report (8).

It has further been investigated that the DAB-destroying ability by rat liver slices was influenced by time intervals and by kinds of dyes. That is, firstly, the ability varied according to the number of hours after injection, gradually approaching the control level but with fluctuating intencity during 48 hours after the time of injection. Similar tendency has been seen in the previous publication (9). Secondly, the ability varied according to the kinds of dyes with which they were loaded. Each dye, except congo red that had effected but slight fluctuation, showed marked fluctuation in DAB-destroying ability during 18 hours after injection.

The above results suggest that these dyes, except congo red which has been said to give but slight influence on the parenchymal cells, attack not only Kupffer cells but also liver parenchymal cells.

In the studies descriced above where the rat liver slices had been employed, it was assured that the DAB-destroying ability could be depressed by the loading of the Kupffer cells with the above mentioned dyes.

There are many observations (10-14) on the blocking of the reticulo-endothelial system which results in the decrease in the functional activities of iron or lipid metabolism, of antibody formation, and of phagocytosis. Furthermore, this system has also been related to the metabolism of vitamin A (21) and the resistance to the growth of transplanted tumors (17-20). Usually, congo red, bromsulfalein, radioactive phosphate had been used for the functional tests of the reticulo-endothelial system, and in those tests the entire body had been employed. In this experiment, however, not the entire body but only the liver slices had been employed, but the results obtained in DAB-destroying ability were analogous.

This fact suggests that Kupffer cells may play an important role in controlling the fate of DAB, particularly destruction, in the living animal. However, it has not been made clear whether, in the destruction of DAB, the Kupffer cells are the main agent or are co-operating with the liver cells.

Recently, Iwase (15) and Sayama (16) have reported that trypan blue inhibited the development of DAB-induced liver cancer. In the present investigation, the influence of trypan blue injection on the DAB-destroying ability of rat liver slices was generally indicative of a hypofunction of the Kupffer cells except in the initial stage where the Kupffer cells suggested some effect of stimulation. Therefore, it is assumed that the inhibition of liver cancer by trypan blue has been based rather on the depression, than on the stimulation, of the functions of the Kupffer cells.

Summary

The studies described in this paper are concerned with the effect of loading Kupffer cells on the destruction of DAB by rat liver slices. India ink, trypan blue, congo red, and carmine were used as agents to 'block' the Kupffer cells.

DAB-destroying ability by rat liver slices were generally depressed by loading of various kinds of dyes. However, the degree of depression varied according to the kinds of dyes or the intervals after the time of loading. The depression of DAB-destroying ability in congo red group was most remarkable and with least fluctuation. Histologically, Kupffer cells in the liver loaded with those dyes revealed various degrees of hypertrophy in each group and stage of loading. The DAB-destroying ability of rat liver slices showed different values according to the degree of loading in the Kupffer cells. The hepatic parenchymal cells revealed no marked change during 48 hours after injection.

From these results it is presumed that Kupffer cells may play an important role in the destruction of DAB in the rat liver.

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要　　旨

ラッテ肝超生切片による分解に対する星細胞封鎖の影響

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p-dimethylaminoazobenzene (DAB) の発癌過程における星細胞の態度を追求するために，肝超生切片による DAB 分解に対する星細胞封鎖の影響を検索した。

すなわち，体重 100～200 g のラッテの腹腔内に，生理的食塩水による 3% 墨汁，1% トリパン青，1% コンゴー赤の注射群および 3% 墨汁と 0.08% カルミンとの重複注射群の 4 群を作り各々 10 cc/kg 体重で使用，一定時間後 (3～48 時間) に屠殺，200 mg の肝超生切片を作り，20 cc の DAB 飽和の Krebs-Ringer 氏液に浮遊させ，O_2-Gas の存在のもとで 37.5℃ の恒温槽内で 30 分間振盪後，直ちに 100℃ 加熱，酵素活性の停止をなし，25% 塩酸アルコール 2 cc を添加，発色により濃度を測定した。同時に，肝組織を Formalin, Susa 固定などを行い星細胞封鎖の程度を組織学的に検索し，DAB 分解能との関係を比較追求した。

その結果，各封鎖物質の種類により DAB の分解能に異った影響をおよぼす。その影響は何れも注射後 18 時間以内に著明である。星細胞封鎖による DAB 分解能への影響は時間の経過と共に動揺を示し，漸次正常に復する傾向を示す。

組織学的には墨汁の場合，星細胞封鎖の程度の強い時，DAB 分解能の抑制も強い傾向を示すが，カルミンとの重複注射群では逆の傾向がみられる。すなわち，何れの場合も肝超生切片による DAB 分解に対する星細胞封鎖の効果は，封鎖物質の種類と時間の経過により変動し，一様ではないが，全般には抑制的である事を明かにした。　(文部省科学研究費による)

[GANN, 49, 199~208; September, 1958]

EFFECTS OF X-RAY IRRADIATION UPON METABOLISM OF DESOXYRIBONUCLEIC ACID AND DESOXYRIBONUCLEASE. II. IN RAT TUMOR

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Since Hevesy's research (1, 2, 3, 4), it is broadly accepted that the outstanding depression of desoxyribonucleic acid metabolism is caused by the ionizing radiation, but few investigators reported on the interrelationship between nucleic acid metabolism and enzyme system concerned with nucleic acid, namely desoxyribonuleic acid depolymerizing enzyme, and the effects of radiation on these enzyme activities of normal as well as tumor tissues.

Greenstein (5, 6, 7, 8, 9) studied on desoxyribonuclease in cancerous hepatic tissue, so called DNase I or neutral DNase, activity of which was measured by viscosimetric method. But recently, Laskowski *et al* (10, 11) and Allfrey *et al* (12, 13, 14) found another type of DNase, DNase II or acid DNase which is generally distributed amoung normal tissues except pancreas. Furthemore, close correlation between DNase activity and turnover rate of the tissue DNA was recognized by means of N^{15} (12). In the present paper biochemical studies on DNA metabolism and DNase activity in a transplantable rat tumor and other tssues of tumor bearing animals and on the effects of X-ray irradiation upon them are presented.

Methods and Materials

The tumor used was a transplantable fibrosarcoma of rat, originally produced by repeated subcutaneous injections of rhodamine B and establised as a transplantable strain (15). It was transplanted subcutaneously to the albino rats with average body weight of 100 g subcutaneausly at the back of animals. Three weeks after transplantation of tumor, the animals were injected intraperitoneally with 1 microcurie of radioactive phosphorus (^{32}P) per body weight, and sacrificed by bleeding 2, 4, 6, 12, 24 and 48 hours after the injection, and tumor tissues, livers and spleens were excised.

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Tissue DNA was prepared according to Mirsky-Pollister (16). After extraction of DNA-histon with 10% NaCl solution, DNA-histon was precipitated by addition of 2 volumes of alcohol and deproteinized by shaking with chloroform-amylalcohol mixture (3 : 1).

The crude DNA was disolved in 0.1 N NaOH solution, freed from contaminating RNA by hydrolysation employing wyatt's procedure (17) and reprecipitated by ethanol acidified with 1 N HCl. The reprecipitation was repeated 5–7 times until the specific activity attained a constant value. The specific activity of the DNA preparation was calculated from phosphorus content determined by Fiske-Subbarrow's method (18) after wet ashing, and radioactivity measured by Geiger-Mullers counter.

Besides the specific activity, relative specific activity of DNA was calculated assuming acid-soluble orthophosphate to be the presursor of phosphate residues in DNA molecule.

$$\text{Relative specific activity} = \frac{\text{specific activity of DNA}}{\text{specific activity of ortho-P}} \times 1000$$

Determination of Desoxyribonuclease Activity :

Every 1 g of the tissue was extracted with 25 ml of Ringer's solution. The DNase activity of the extract was determined by measuring the rate of release of acid soluble diphenylamine reactive substance (12) from highly polymerized thymonucleic acid prepared from calf thymus according to Mirsky-Pollister (16). The reaction vessel contained the following components; substrate DNA 1000 microgram (0.5 ml); enzyme solution 1 ml; Walpole's acetate buffer for pH 4 to 7 or borate buffer for pH above 7, 1 ml.; final volume being made up to 3 ml. The reaction was allowed to proceed for 60 minutes at 37°C; it was stopped by the addition of 1 ml of 3.6 M trichlor-acetic acid. The precipitated protein was removed by centrifugation at 4000 r. p. m. for 15 minutes, an aliquot of the supernatant fluid was heated with addition of 3 ml of Dische's reagent (19) in a boiling water bath for 20 minutes and the difference between the extinction values at 590 mμ and 650 mμ was determined.

Enzyme unit was expressed by the amount of depolymerized DNA in microgram per mg dry tissue per hour at 37°C.

The animals were exposed to a single whole body X-ray irradiation of 300 r dosage, the dose rate being 67 r/min.

The radiation factors were; 200 kVp 20 mA, and filtered with 1.0 mm thick cupper plate and 0.5 mm thick aluminum plate.

As for the irradiated groups, the experiments were carried out 24 hours after the X-ray irradiation.

In vitro incubation——slices of the tumor were incubated in Krebs Ringer

phosphate solution of pH 7.4 which was fortified by the addition of glucose (0.2% final), ATP (6×10^{-3} M), and of amino acid mixture (0.1% final) composed of 17 kinds of amino acids, where 40 mg of the tissue by wet weight was received in 1 ml of the solution.

The amino acid mixture was composed of arginine 2.8%, histidine 1.9%, lysine 5.0%, tryptophan 4.1%, threonine 4.5%, leucine 14.6%, valine 8.1% phenylalanine 6.0%, tyrosine 3.7%, methionine 4.0%, cystine 0.2%, serine 6.9%, glutamic acid 1.1%, aspartic acid 7.2%, alanine 6.4%, glycine 0.3%, and proline 4.7%.

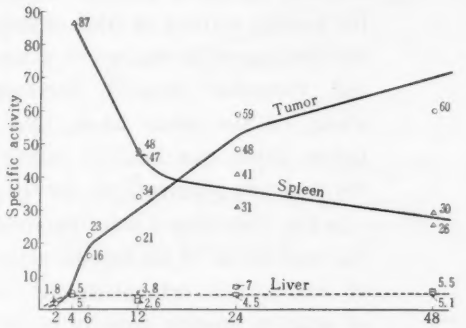

Fig. 1. The time course in hours of the specific activities of the DNA of tumor, and spleen and liver of the tumor bearing animals, which received intraperitoneal administration of ^{32}P, 1 microcurie/g body weight.

Results and Discussion

Metabolism of DNA—— In the present experiment, the DNA used was prepared by the method of Mirsky-Pollister from various tissues.

Since the DNA extracted by this method possesed higher molecular weight than that obtained by the method of Kay *et al* (20), the further purification of DNA by NaOH and HCL (17) was more easily performed in

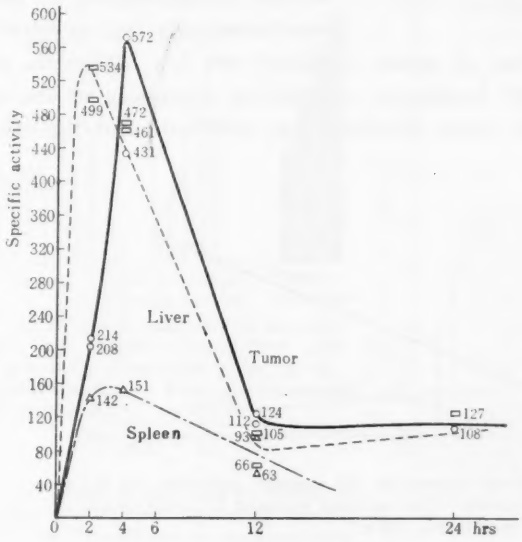

Fig. 2. The time course of the specific activities of the acidsoluble orthophosphates of the above mentioned tissues.

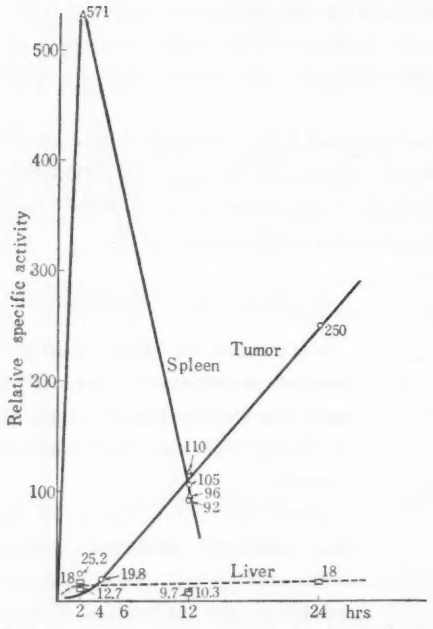

Fig. 3. The time course of the relative specific activities of the DNA of the tissues. The value of the relative specific activity was expressed as follows:

$$\frac{\text{Specific activity of DNA}}{\text{Specific activity of ortho-P}} \times 1000$$

the DNA by Mirsky-Pollister method than that of the latter.

The Specific activities of DNA of sarcoma, spleen and liver of the tumor bearing animals are shown in Fig. 1. It is very outstanding that each of the three tissue examined represents its characteristic metabolic rate and metabolic pattern. Namely, the specific activity of DNA of spleen was the highest at the end of 4 hours and thereafter rapidly decreased, while, on the other hand, that of tumor tissue was initially relatively low and was progressively elevated.

In Fig. 2 and Fig. 3 were illustrated the time course of the specific activity of acid-soluble orthophosphate, and of relative specific activity of DNA from several tissues, respectively.

Contrary to the specific activities of DNA the specific activities of acid-soluble orthophosphate of the liver was exceedingly high at initial stage (after 2 hours), whereas that of spleen remained very low. When the animals received whole body X-ray irradiation, outstanding depression of the specific activities of DNA of all the tissue examined was observed. But the character-

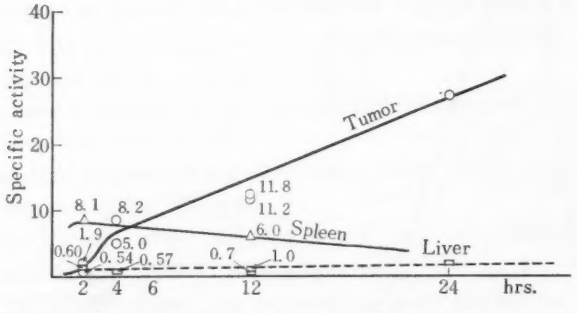

Fig. 4. The time course of the specific activities of the DNA of the tissues. The animals received 24 hours before whole body X-irradiation, 300 r.

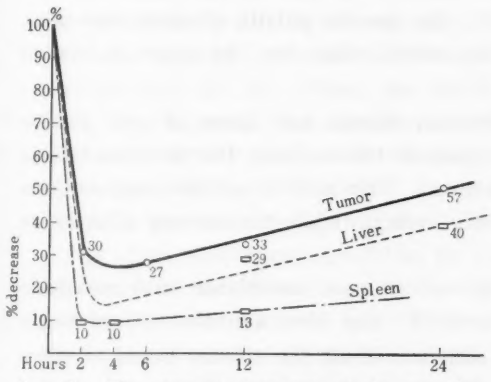

Fig. 5. The percent decrease of the specific activities of the DNA caused by the whole body X-irradiation.

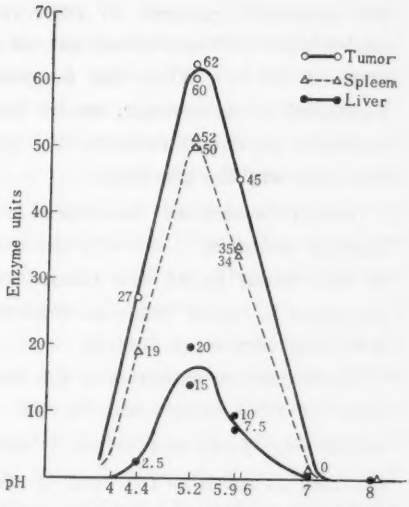

Fig. 6. The pH activity curves of the DNase II activity. The enzyme units are expressed as depolymerized DNA in micro-gr/mg dry tissue/hrs at 37°C incubation.

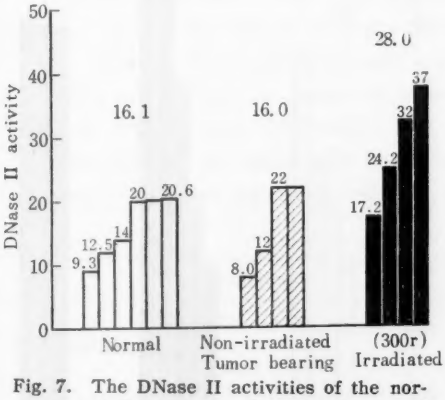

Fig. 7. The DNase II activities of the normal liver tumor bearing liver and whole body X-irrdiated liver of the tumor bearings. After 24 hours of X-irradiation.

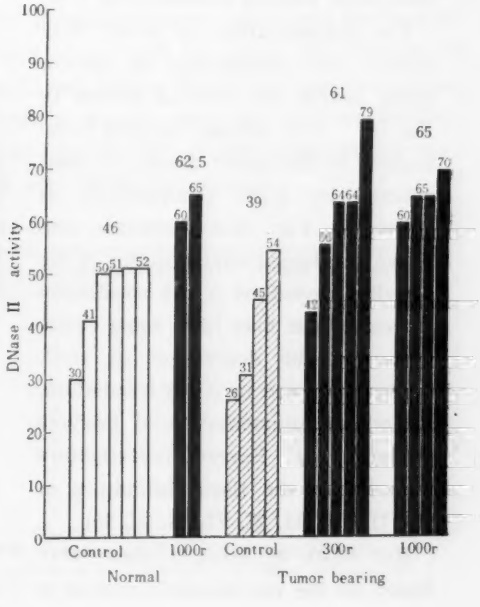

Fig. 8. The DNase II activities of spleens of tumor bearing and non-tumor bearing animals, X-irradiated or non-irradiated.

stic metabolic patterns of DNA remained unchanged. The speci fic activity of acid-soluble orthophosphate as not affected at all by X-ray irradiation with the dosis of 300 r. When the de pression of the specific activities of DNA was expressed in percentage, as in Fig. 5, the specific activity of spleen was most markedly decreased down to 10% of the control value, but the value of tumors was only slightly affected.

Desoxyribonuclease Activities in Tumor, Spleen and Liver of the Tumor bearing Animals. —— Of the two types of DNase, only the so-called DNase II was found in all the tissues examined, The activity of this enzyme was strongest in tumor tissue as regards dry weight, while the enzyme activity in liver was very weak (Fig. 6).

These enzyme activities of the tissue were in good accordance with metabolic rates of DNA determined by the use of ^{32}P. But when animals received single whole body X-ray irradiation, DNase activities of all the tissues examined were elevated as much as two fold at the end of 24 hours after X-ray irradiation.

The DNase II activities of the spleen and liver in tumor bearing animals were were similar to those of non-tumor bearing animals (Fig. 7, 8).

The augmentation by X-ray irradiation was conspicuous in spleen, being nearly two fold as shown in Fig. 8. The DNase activity was highest in the tumor tissue, but least affected by X-ray irradiationes as shown in Fig. 9. Therefore, the above mentioned augmentation of the activity caused by X-ray irradiation in each tissue may have some correlation to the depression of DNA turnover caused by X-ray irradiation.

Mode of Augmentation of Enzyme Activity. —— Several investigators reported on the tissue inhibitors of the DNase (11, 21, 22, 23, 24, 25).

But most of these studies were based on the viscosimetric method or

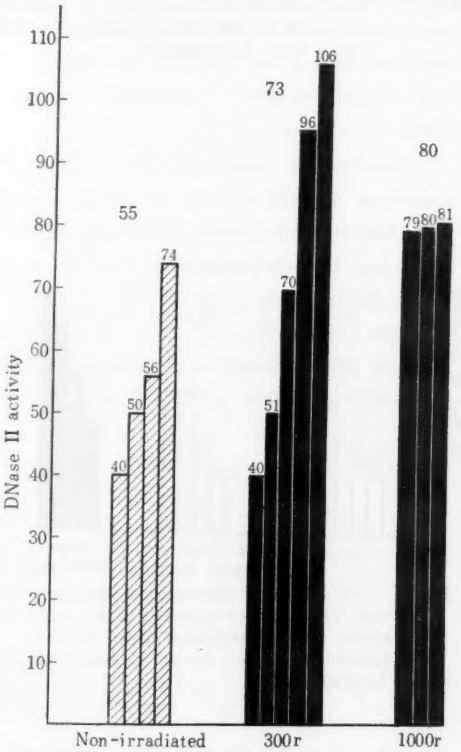

Fig. 9. The DNase II activities of the tumor animals receiving or not receiving the whole body X-irradiation.

on DNase I activity and raised certain questions. It failed to find the natural inhibitors specific for DNase II. In several experiments with irradiated and nonirradiated tissues (spleen, tumor) the observed activity of the mixed tissues was equall to the sum of the activities of the tissues measured individually.

It was recently reported that coexisting RNA inhibited DNase activity in bacterial cell (26, 27). When the tissue was incubated with 100 microgram of RNA, approximately 25% inhibition of tissue DNase activity was observed, and incubation with crystalline ribonuclease at pH 5.2, tissue DNase II activity was only slightly affected. Therefore, it may be thought that the RNA was not concerned in the inhibition of the animal tissue DNase II activity.

The whole body X-ray irradiation on the tumor bearing animals resulted in the depression of the ^{32}P incorporation in the tumors and spleen, while the DNase II activities in both organs were increased. The similar rise of DNase II activities in the spleen and thymus in normal rats have been previously reported by Fellas et al. (28), and Okada et al. (29, 30, 31). The cause of such a rise of DNase II activities in these tissues have been investigated in *in vitro* experiment with tissue slices. Since it was reported that biosynthesis of amylase in pancreas slices *in vitro* was considerably enhanced after the animal received whole body X-ray irradiation (32, 33), some questions arosed as to whether the augmentation of DNase activity caused by X-ray irradiation is due to the real increase of enzyme synthesis or not. The tumor tissue slices were incubated after whole body X-ray irradiation in Krebs-Ringer phosphate buffer which was fortified by the addition of glucose, ATP and amino acid mixture composed of 17 kinds of amino acids found in casein hydolysate, and DNase II activity was determined after definite time intervals. The control groups were out without addition either of ATP or amino acid mixture, or kept at 0°C. Similar experiments were conducted under anaerobic condition or with tissue homogenate.

Table. 1. In vitro incubation of tumor slices

Condition		Incubation time (hours)	DNase II activity
Aerobic	Control	0	40
	Complete system	2	40
		18	45
Anaerobic	Complete system	2	40
		18	60
	Without addition of amino acid mixture and glucose	2	51
		18	75

After in vitro incubation under the above mentioned condition, the DNase II activity was measured. Enzyme units are expressed as depolymerized DNA in mirogram/mg tissue/hour at 37°C The complete system contained glucose (0.2% final) ATP ($6\times10M$), amino acid mixture and Krebs-Ringer phosphate buffer, pH 7.4.

Under aerobic condition, slight increase of enzyme activity was observed after 18 hours incubation, about 12% increase (Table 1). On the other hand, under anaerobic condititon more increse was obtained after 18 hours incubation, about 50% increase. This suggested that such a rise of the enzyme activity may not be due to the enzyme synthesis, but the slight increase of the enzyme activity in the irradiated tumor tissues under prolonged anaeroblic incubation might have resulted from the degradation of the cells. Notable increase of enzymatic activities were reported after anaerobic incubation, such as outstanding increase of mitochondrial ATPase activity after aging or treating with 2.4-dinitrophenol (34). It is also reported that increase of some bacterial amylase followed anaerobic lysis of B. subtilis (35). But DNase activity was not increased by the treatment with 2.4-dinitrophenol, 10^{-2M} to 10^{-3M} of it, rather showing a slight inhibition. Therefore, it may be thought that the DNA in several tissues was not always strictly metabolically stable, but being newly synthesized and to some extent brocken down by enzymatic process, and the depression of DNA synthesis in normal tissues as well as in the tumors resulted following irradiation, possibly accompanying with the enhanced enzymatic depolymerisation process, found by the rise of DNase II activity.

Summary

1) The metabolism of DNA of rat tumor, and liver and spleen of the tumor bearing animals were studied with the use of ^{32}P, the charcteristic time course of the specific activity of the DNA, prepared by the method of Mirsky and Pollister and purified by Wyatt's procedure, was observed in each of the tissues.

2) The DNase II activity was measured by the method of Allfrey and Mirsky, and a close correlation of the DNase II activity was observed with the metabolic rates of DNA of each tissue by the use of ^{32}P, namely highest in tumor, and low activity in liver.

3) Following whole body X-ray irradiation, the metabolic rates of DNA of each tissue was depressed conciderably, and on the contrary, the DNase II activity was enhanced after irradiation, namely the DNase II activity of the spleen was increased about two fold.

4) On the mechanism of augmentation of DNase II activity caused by the X-ray irradiation some *in vitro* experiments were carried out, paticulary from the standpoint of the enzyme protein synthesis.

Acknowledgement

The authers wish to express their thanks to Miss K. Shibata, Department of Radiology, Faculty of Medicine, University of Tokyo, for her assistance.

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要　旨

ラット腫瘍における DNA 代謝，並びに DNase 活性におよぼす X 線の作用

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移植性腫瘍および垣癌動物の肝，脾における DNA 代謝を放射性燐 P^{32} を用いて検索し，これにおよぼす放射線の著明な抑制低下を認める一方これらの組織における DNA 解合酵素活性を測定し，DNA 代謝の rate とこの DNase 活性の強さとがよし一致していること，またこの酵素活性はレ線照射に依って著明に増強することを認めた。DNA 代謝は P^{32} を 1 μc/gm の量で腹腔内注射し，その後 2, 4, 6, 12, 24 および 48 時間後に組織の DNA を Mirsky-Pollister 法で抽出精製した後，その比放射能 cpm/DNApr を測定し，腫瘍，脾が高く，肝が低い値を示した。

レ線照射（全身照射）300 r 24 時間後にこれ等の値は著しく低下し，脾は 1/10 に，肝および腫瘍は各々 1/3 に低下した。DNA 解合酵素活性は何れの組織はおいても DNase II と呼ばれる所謂酸性 DNase 活性のみが認められ，腫瘍，脾に強く，各々 50，および 45 酵素単位を示すか，肝は低く 15 単位であった。一方上記照射後，24 時間後に酵素活性は増強し，脾に著しく，約 2 倍となった。この活性増強の機序について，酵素蛋白の合成の増加があるか否かについて，in vitro での slice の incubation を行ったが，酵素蛋白の合成の増加は認められなかった。

[GANN, 49, 209~222; September, 1958]

THE EFFECT OF MITOMYCIN C ON THE GROWTH OF A VARIETY OF RAT AND MOUSE TUMORS

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In 1956 Hata and his co-workers[1,2] reported that *Streptomyces caespitisus* produced new antibiotic mitomycins that had a strong activity against various microbes and Ehrlich carcinoma. They further reported that mitomycin complex consisted of many active fractions, from which two main substances, A and B, could be isolated in crystalline form.

Wakaki and his co-workers[3] thereafter reported that a new bluish-violet fraction called mitomycin X was isolated in the process of culturing the strain producing mitomycin. This mitomycin X has been renamed mitomycin C.

It was reported from several laboratories[4,5] in Japan that mitomycin C was strongly active not only against Ehrlich carcinoma, but also against Walker carcinosarcoma, Crocker sarcoma 180 and Yoshida sarcoma.

Our observations on the effect of mitomycin C on a variety of transplantable rat and mouse tumors have been reported briefly[6,7], and they are new presented in this paper in more detail.

Materials and Methods

The tumors used in the present study are as follows: Yoshida sarcoma, Diploid Hirosaki sarcoma, Tetraploid Hirosaki sarcoma, Takeda sarcoma, Usubuchi sarcoma, Ascites hepatoma 130, Ascites hepatoma 7974 in hybrid rats, Lymphatic leukemia SN36 and Ehrlich carcinoma in dd mice.

The history and biological properties of Yoshida sarcoma and Ehrlich carcinoma are well-known. Brief descriptions of other tumors used in this report are as follows.

Diploid Hirosaki sarcoma[8]: This ascites tumor originated in 1951 in the cervical lymph node of a hybrid rat in our laboratory. Biological and cytological properties of this tumor are very similar to those of Yoshida sarcoma. The chromosome numbers of this tumor distrobute in a relatively wide range and have a peak at about 40.

Tetraploid Hirosaki sarcoma[8]: This type of Hirosaki sarcoma developed spontaneously in the course of the intraperitoneal transplantation of Diploid Hiro-

saki sarcoma from generation to generation. The chromosome numbers of the tumor fluctuate in a wide range showing a peak at about 70. Biological properties of this tumor are very similar to those of the diploid type.

Takeda sarcoma[9]: This ascites tumor was discovered in a hybrid rat by Takeda (1952) of Hokkido Univerity. In the course of the successive intraperitoneal transplantations, the characteristics of this tumor gradually changed from the original fibrosarcomatous structure to that of reticulum cell sarcoma.

Usubuchi sarcoma[10]: This ascites tumor originated in 1952 from our laboratory as a spindle cell sarcoma caused by methylcholanthrene injection.

In the course of the successive intraperitoneal transplantations, the characteristics of this tumor changed gradually to those of reticulum cell sarcoma as Takeda sarcoma.

Ascites hepatoma 130 and 7974[11]: Both of these tumors were originally brought about in 1952 in Sasaki Institute by intraperitoneal transplantation of DAB hepatomas in rats.

Lymphatic leukemia SN36[12]: This ascites tumor originated in 1957 in the same institute from a dd mouse by subcutaneous successive injections of nitromin.

The animals used in our experiments were hybrid rats weighing about 100g and mice weighing about 20g, and the methods employed in the chemotherapy studies were divided into two groups as follows:

1) Ascitic forms of the tumors: The first intraperitoneal injections of 50-100 mcg/kg of mitomycin C were administered 48 hours after intraperitoneal transplantation of the tumor. Injections were continued intraperitoneally once a day for 3-33 days, in most cases at maximum tolereted doses (500-1000 mcg/kg) for 7 days, at middle doses (200-500 mcg/kg) for 15 days and at smaller doses (50-200 mcg/kg) for 30 days.

Ascites was examined daily in rats and every 4 days in mice by the smear preparation coloured with Giemsa's stain. In each group about 10 animals were tested, and they were investigated histologically, after they died. The animals were regarded as being cured, when no tumor was found anywhere 100 days after the transplantation. The positive effect on life-prolongation was admitted to be present, when the mean survival period in the treated animals amounted to about twice of those of the controls.

2) Solid forms of the tumors: Diploid and Tetraploid Hirosaki sarcoma were used for the experiment. The first intraperitoneal injections of 200-500 mg/kg of mitomycin C were given 3-10 days after subcutaneous transplantation of the tumor. Therapeutical injections were continued intraperitoneally once a day for 7-15 days, that is, at 500 mcg/kg a day for 7 days and at 200 mcg/kg a day for 15 days.

The progress of the tumors in the animals was recorded graphically by mea-

suring them in two diameters with calipers every 5 days for 20 days after tumor transplantation.

Results

A. Effect on ascitic forms of the tumors.

1. Effect on Yoshida sarcoma (Fig. 1).

Forty-five control animals died from tumor invasion between 6-12 days after the intraperitoneal transplantation. The mean survival period was 8.6 days. No spontaneous regression was seen.

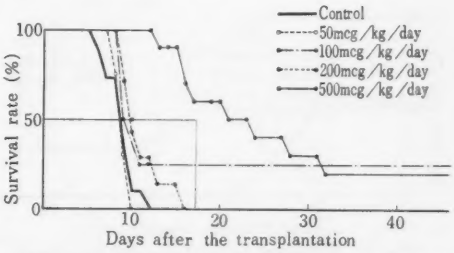

Fig. 1. Effect of intraperitoneal injections of mitomycin C on 2-day-old ascitic forms of Yoshida sarcoma, showing a positive effect on life-prolongation at 500 mcg/kg/day.

Three groups consisting of 4, 4 and 7 rats were treated with daily doses of 50 mcg/kg, 100 mcg/kg and 200 mcg/kg of mitomycin C respectively.

Tumor cells in the ascites decreased in number, but in every group neither inhibition of tumor formation nor prolongation of survival days was seen. Ten rats, which were treated with daily doses of 500 mcg/kg, showed a prolongation of survival period, but after all most of them died from tumor invasion.

2. Effect on Diploid Hirosaki sarcoma (Fig. 2).

Out of 250 rats, which were used for the successive intraperitoneal transplantations of the tumor without any treatment, 98 per cent died from tumor invasion. Most of them died between 6-18 days, and the mean survival period was 7.8 days.

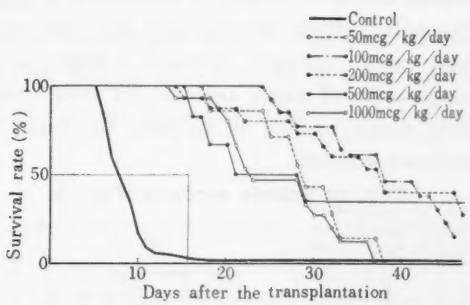

Fig. 2. Effect of intraperitoneal injections of mitomycin C on 2-day-old ascitic forms of Diploid Hirosaki sarcoma, showing a positive effect on life-prolongation at 50 mcg/kg/day.

Three groups consisting of 7, 13 and 15 rats were treated with daily doses of 50 mcg/kg, 100 mcg /kg of mitomycin C respectively.

Tumor cells in the ascites decreased in number or disappeared within several days and survival period prolonged markedly, but recidivations were seen in most cases after discontinuing the treatment.

Two groups consisting of 6 and 15 rats were treated with daily doses of 500

mcg/kg and 1000 mcg/kg of mitomycin C respectively. Tumor cells disappeared from the ascites within several days, and most cases showed a marked prolongation of survival period. Autopsy revealed a slight or no tumor formation in cases of administration of larger amounts, in some cases of which atrophy of the spleen or early death seemed to indicate the toxity of the drug.

3. Effect on Tetraploid Hirosaki sarcoma (Fig. 3).

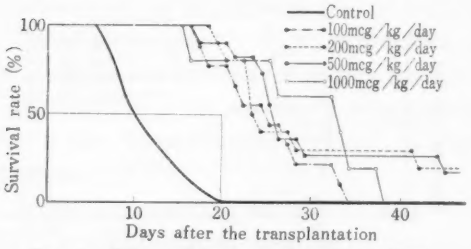

Fig. 3. Effect of intraperitoneal injections of mitomycin C on 2-day-old ascitic forms of Tetraploid Hirosaki sarcoma, showing a positive effect on life-prolongation at 100 mcg/kg /day.

Out of 580 rats, which were used for the successive intraperitoneal transplantations of this tumor without any treatment, 99 per cent died from tumor invasion. Most of them died between 6-20 days, and the mean survival period came to 9.6 days.

Two groups consisting of 9 and 10 rats were treated with daily doses of 100 mcg/kg and 200 mcg/kg of mitomycin C respectively. Tumor cells in the ascites decreased in number or disappeared within several days, and the survival period prolonged markedly, but recidivation occurred in most cases after stopping the treatment.

Two groups consisting of 11 and 5 rats were treated with daily doses of 500 mcg/kg and 1000 mcg/kg of mitomycin C respectively. Tumor cells in the ascites disppeared within several days and the survival period markedly prolonged in most cases. Autopsy revealed a slight or no tumor formation in cases of administration of larger amounts, in some cases of which atrophy of the spleen or early death seemed to indicate the toxity of the drug as in cases of Diploid Hirosaki sarcoma.

4. Effect on Takeda sarcoma (Fig. 4).

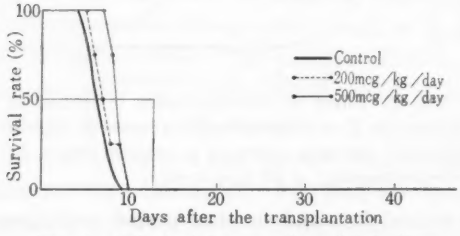

Fig. 4. Effect of intraperitoneal injections of mitomycin C on 2-day-old ascitic forms of Takeda sarcoma, showing no effect on life-prolongation at 500 mcg/kg/day.

Sixty-one control animals died from sarcomatous peritonitis with a large quantity of haemorrhagic asites between 5-9 days after intraperitoneal transplantations. The survival period was 6.3 days on the average. No spontaneous regression was seen.

Two groups consisting of 4 and 4 rats were treted wit daily doses of 200 mcg/kg and 500 mg/kg of mito-

mycin C respectively. Tumor cells in the ascites slightly decreased in number, but no prolongation of survival period took place. Then, 2 rats were administered with daily doses of 1000 mcg/kg and 2000 mcg/kg of mitomycin C respectively. Neither inhibition of tumor growth nor prolongation of survival period was found in both cases.

5. Effect on Usubuchi sarcoma (Fig. 5).

Out of 320 rats, which were used for the successive intraperitoneal transplantations of the tumor without any treatment, 94 per cent died from tumor invasion. Most of them died between 8-12 days and the mean survival period was 14 days.

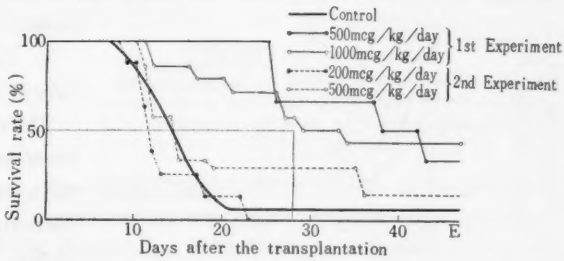

Fig. 5. Effect of intraperitoneal injections of mitomycin C on 2-day-old ascitic forms of Usubuchi sarcoma, showing a positive effect on life-prolongation at 500 mcg/kg/day in the first experiment.

Two kinds of experiment were carried out at interval of about 8 months. In the first experiment two groups consisting of 6 and 14 rats were treated with daily doses of 500 mcg/kg and 1000 mcg/kg of mitomycin respectively. Tumor cells disappeared from the ascites after several days. A remarkable prolongation of survival period occurred in most cases.

In the second experiment two groups consisting of 8 and 7 rats were treated with daily doses of 200 mcg/kg and 500 mcg/kg of mitomycin C respectively. Tumor cells in the ascites decreased in number, but no prolongation of survival period was seen in most cases.

The different results in two experiments mentioned above might have been caused by a small difference in proliferetive property of the same tumor at different stages.

6. Effect on Ascites hepatoma 130 (Fig. 6).

Fifty-two control animals died from tumor invasion between 7-15 days after intraperitoneal transplantations. The survival period was 11.5 days on the average. No spontane-

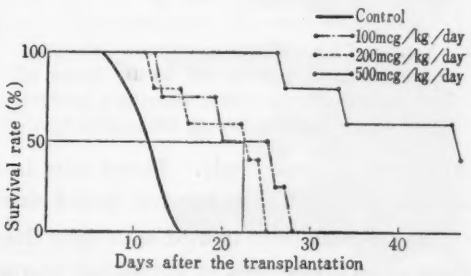

Fig. 6. Effect of intraperitoneal injections of mitomycin C on 2-day-old ascitic froms of Ascites hepatoma 130, showing a positive effect on life prolongation at 100 mcg/kg/day.

ous regression occurred.

Two groups consisting of 4 and 5 rats were treated with daily doses of 100 mcg/kg and 200 mcg/kg of mitomycin C respectively. Tumor cells in the ascites decreased in number, and a prolongation of survival period was seen in most cases.

Other 5 rats were treated with daily doses of 500 mcg/kg of mitomycin C. Tumor cells disappeared from the ascites after several days. A remarkable prolongation of survival period and inhibition of tumor growth were seen in all cases.

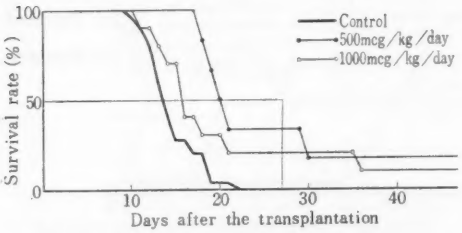

Fig. 7. Effect of intraperitoneal injections of mitomycin C on 2-day-old ascitic forms of Ascites hepatoma 7974, showing no effect on life-prolongation at 1000 mcg/kg/day.

7. Effect on Ascites hepatoma 7974 (Fig. 7).

Thirty-six control animals died from tumor invasion between 10-22 days after intraperitoneal transplantations. The mean survival period was 13.6 days. No spontaneous regression was seen.

Two groups consisting of 6 and 10 rats were treated with daily doses of 500 mcg/kg and 1000 mcg/kg of mitomycin C respectively. Tumor cells decreased in number and inhibition of tumor growth occurred in most cases, but no obvious prolongation of survival period was present.

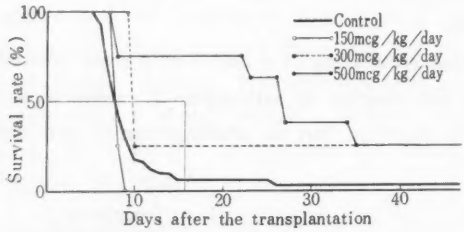

Fig. 8. Effect of intraperitoneal injections of mitomycin C on 2-day-old ascitic forms of Lymphatic leukemia SN36, showing a positive effect on life-prolongation at 500 mcg/kg/day.

8. Effect on Lymphatic leukemia SN36 (Fig. 8).

Out of 66 control mice, 67 per cent died from tumor invasion between 6-15 days after intraperitoneal transplantations causing a leukemic manifestations in blood. The mean survival period was 7.5 days.

Two groups consisting of 4 and 4 mice were treated with daily doses of 150 mcg/kg and 300 mcg/kg of mitomycin C respectively. Tumor cells in the ascites showed no obvious change and no prolongation of survival period was observed.

Other 8 mice were treated with daily doses of 500 mcg/kg of mitomycin C. In those cases tumor cells in the ascites decreased or disappeared and their survival periods prolonged remarkably.

9. Effect on Ehrlich carcinoma (Fig. 9).

Forty-eight mice died from tumor invasion between 13-22 days after intrapreito-

neal transplantations. The mean survival period was 18 days and no spontaneous regression took place.

Four mice were treated with daily doses of 125 mcg/kg of mitomycin C. Tumor cells in the ascites showed no obvious change and no prolongation of survival period was seen.

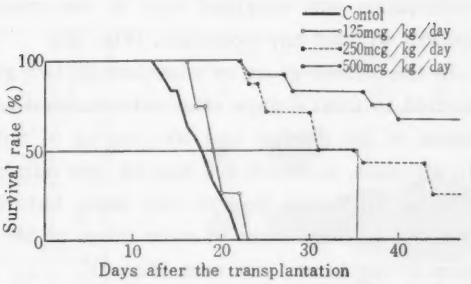

Fig. 9. Effect of intraperitoneal injections of mitomycin C on 2-day-old ascitic forms of Ehrlich carcinoma, showing a positive effect on life-prolongation at 250 mcg/eg/day.

Two groups consisting of 8 and 12 mice were treated with daily doses of 250 mcg/kg and 500 mcg/kg of mitomycin C respectively. Tumor cells decreased in number or disappeared, and a marked prolongation of survival period was seen.

B. Effect on solid forms of the tumors.

1. Effect on Diploid Hirosaki sarcoma.

In the first group of experiments 6 rats were started to treat 3 days after subcutaneous transplantation of the tumor. 500 mcg/kg of mitomycin C were injected daily into the abdominal cavity for 7 days. Ten days after the beginning of the treatment a complete regression of the tumor was seen in all cases and a

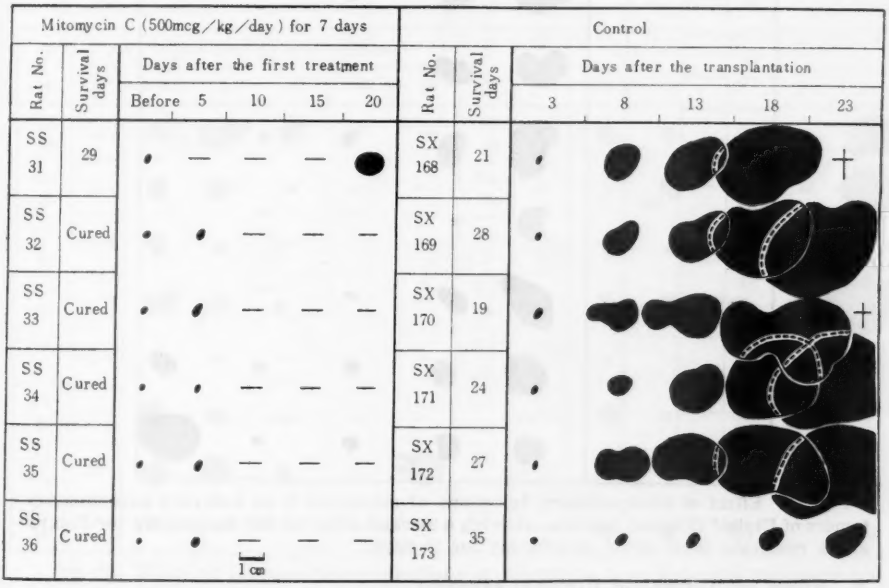

Fig. 10. Effect of intraperitoneal injections of mitomycin C on 3-day-old subcutaneous tumors of Diploid Hirosaki sarcoma, showing a marked effect at 500 mcg/kg/day for 7 days.

recidivation was observed only in one case. Six control rats died from tumor growth without any exception. (Fig. 10).

In the second group of experiments two groups consisting of 3 and 7 rats were started to treat 6 days after subcutaneous transplantation of the tumor with daily doses of 200 mcg/kg and 500 mcg/kg of mitomycin C respectively for 7–15 days. In all cases, in which 200 mcg/kg was administered for 15 days, a moderate inhibition of tumor growth was seen, but the animals died from recidivation of tumor. In most cases of daily doses of 500 mcg/kg for 7 days a complete regression of the tumor was seen (Fig. 11).

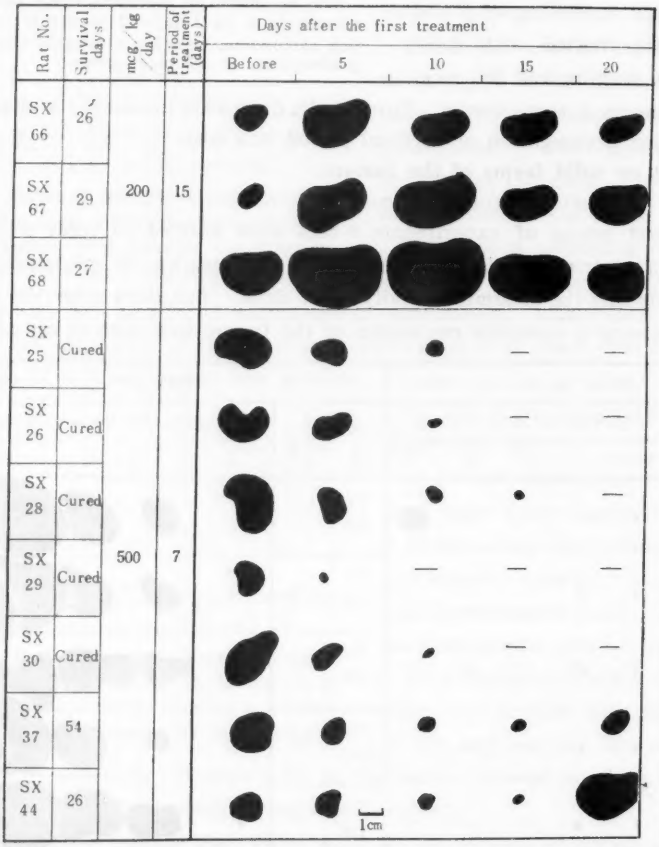

Fig. 11. Effect of intraperitoneal injections of mitomycin C on 6-day-old subcutaneous tumors of Diploid Hirosaki sarcoma, showing a marked effect at 500 mcg/kg/day for 7 days and a moderate effect at 200 mcg/kg/day for 15 days.

2. Effect on Tetraploid Hirosaki sarcoma.

In the first group of experiments 6 rats were started to treat 3 days after

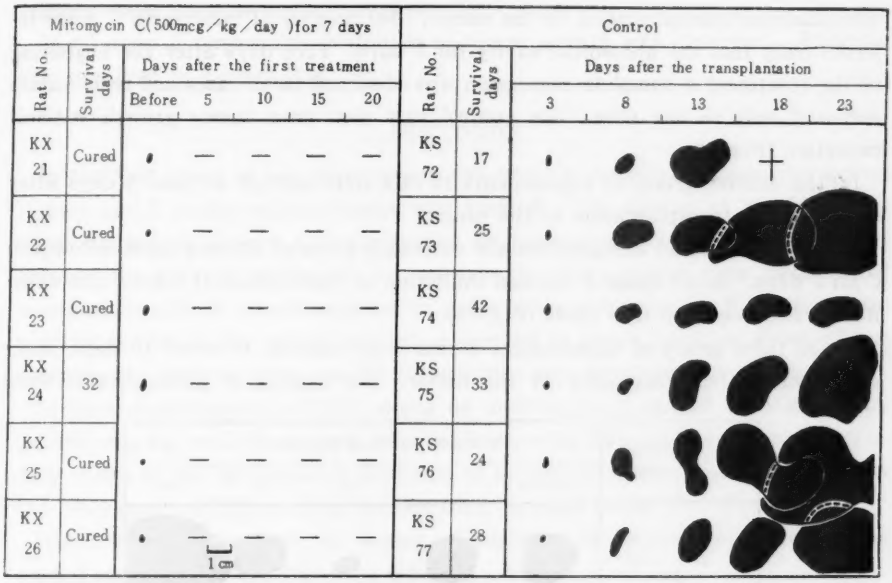

Fig. 12. Effect of intraperitoneal injections of mitomycin C on 3-day-old subcutaneous tumors of Tetraploid Hirosaki sarcoma, showing a marked effect at 500 mcg/kg/day for 7 days.

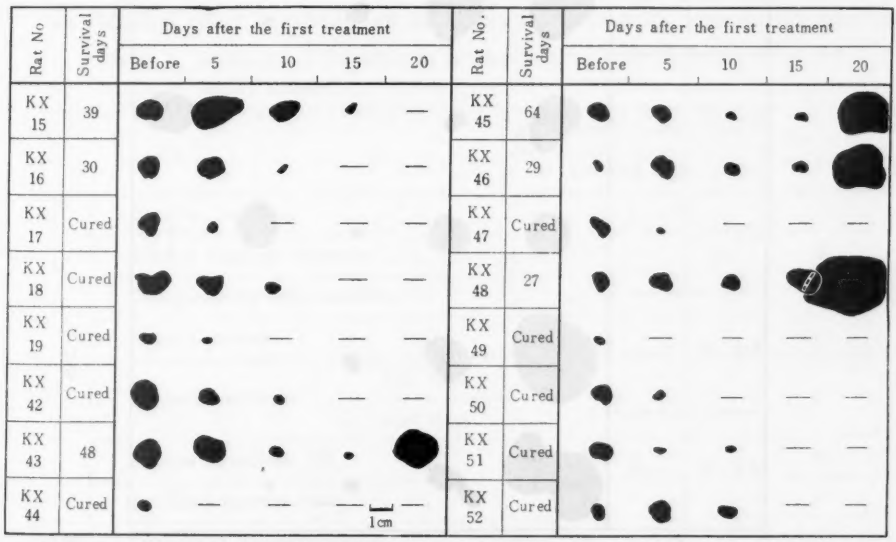

Fig. 13. Effect of intraperitoneal injections of mitomycin C on 5-day-old subcutaneous tumors of Tetraploid Hirosaki sarcoma, showing a marked effect at 500 mcg/kg/day for 7 days.

sebcutaneous transplantation of the tumor. 500 mcg/kg of mitomycin C were injected daily into the abdominal cavity for 7 days. Five days after the beginning of the treatment a complete regression was observed in all cases and recidivation occurred only in one case. Six control rats died from tumor growth without exception (Fig. 12).

In the second group of experiments 16 rats were started to treat 5 days after subcutaneous transplantation of the tumor.

They were injected intraperitoneally with daily doses of 500 mcg/kg of mitomycin C for 7 days. In all cases a marked inhibition or destruction of tumor was seen. Recidivation occured in 6 cases (Fig. 13).

In the third group of experiments 6 rats were started to treat 10 days after subcutaneous transplantation of the tumor. 500 mcg/kg of mitomycin C were

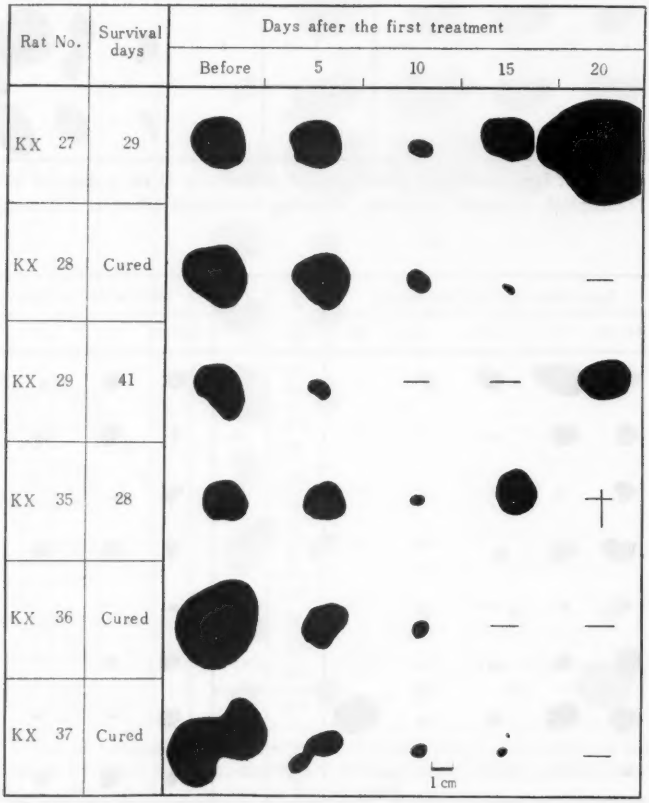

Fig. 14. Effect of intraperitoneal injections of mitomycin C on 10-day-old subcutaneous tumors of Tetraploid Hirosaki sarcoma, showing a marked effect at 500 mcg/kg/day for 7 days.

injected daily into the abdominal cavity for 7 days.

In all cases a remarkable inhibition of tumor growth was observed. Recidivation occurred in 3 cases (Fig. 14).

Discussion

In the first group of our experiments the effect of mitomycin C on 7 rat ascites tumors and 2 mouse ascites tumors was studied. The degree of the inhibitory effect on ascites tumors may be graded by the prolongation of survival period, because the survival period of the animals, in which tumor were transplanted intraperitoneally, is almost constant in cases of the same tumor. When the mean survival period of the treated animals amounted to about twice of those of the controls, the positive effect on life-prolongation was admitted to be present.

In these experiments the daily doses of motomycin C ranged from 50 mcg/kg to 1000 mcg/kg, and the minimal effective doses were different in each tumor. The daily doses of 500 mcg/kg were admitted to be the maximum tolerable doses, as the daily doses of 1000 mcg/kg seemed toxic in some cases.

Based on these reasons the degree of inhibition of ascites tumor growth was graded according to the following scheme:

+ : Slight effect, indicating an inhibitory effect in daily doses of 500 mcg/kg (the maximum tolerable doses).

: Moderate effect, indicating an inhibitory effect in daily doses of 200 mcg/kg.

: Marked effect, indicating an inhibitory effect in daily doses of 100 mcg/kg.

Table 1. Summary of the effect of mitomycin C on 9 ascitic forms of rat and mouse tumors.

			MitomycinC (mcg/kg/day)					Grade of the effect
			50	100	200	500	1000	
Rat	Yoshida sarcoma		−	−	−	+		+
	Diploid Hirosoki sarcoma		+	+	+	+	+	###
	Tetraploid Hirosaki sarcoma			+	+	+	+	##
	Takeda sarcoma				−	−	−	−
	Usubuchi sarcoma	1 st Exp.				+	+	±
		2 nd Exp.			−	−		
	Ascites hepatoma 130			+	+	+		##
	Ascites hepatoma 7974					−	−	−
Mouse	Lymphatic leukemia SN 36			−	−	+		+
	Ehrlich carcinoma			−	+	+		##

卌 : Striking effect, indicating an inhibitory effect in daily doses of 50 mcg/kg.

The inhibitory effect of mitomycin C on 7 rat ascites tumors and 2 mouse ascites tumors is summarized in Table 1. Mitomycin C showed an inhibitory effect on 7 out of 9 ascites tumors. Above all, it was markedly effective on both strains of Hirosaki sarcoma and Ascites hepatoma 130.

It is interesting in these experiments that the effects of mitomycin C on the tumors of the same kind are not just the same, as shown in Hirosaki sarcoma and Yoshida sarcoma, in Ascites hepatoma 130 and 7974, and in Takeda sarcoma and Usubuchi sarcoma.

Sugiura and others[13,14,15,16] reported that the responses of various tumors to a given agent were remarkably different. By their broad experiments it was furthermore demonstrated that the responses of tumors of the same kind to a given agent were not always the same. In Japan Yoshida[17] has shown that the responses of various ascites hepatomas to nitromin were strikingly different. Oboshi[18] also reported the different responses to nitromin in Yoshida sarcoma, Hirosaki sarcoma and his own nitromin-resistant Lymphosarcoma. The different responses of tumors of the same kind to mitomycin C may be of similar nature as reported previously by these authors.

Sugiura and others[16] have reported recently that fumagilin was the most effective among fifteen purified or crystalline antibiotics tested. Although the tumors used in our experiments are different from those used in Sugiura's experiment, mitomycin C showed a broad spectrum which might be compared with that of fumagiline.

Sugiura and others further reported that actinomycin D, actinomycin J, sarcomycin and carzinophilin had a complete destructive effect on all three ascites tumors tested, but these antibiotics had practically no effect on solid tumors with several exceptions. The deta seem to be significant, as our experiments of mitomycin C were carried out mostly on ascites tumors. The effect of mitomycin C against two strains of Hirosaki ascites sarcoma and Ascites hepatoma 130 in minimal doses of 50–100 mcg/kg 48 hours after transplantation, however, seems to be different from those of actinomycin and others.

The second group of experiments, which showed a marked effect of mitomycin C on solid tumors, may give an adequate answer to the question discussed above.

Summary

1) Mitomycin C showed a broad anti-tumor spectrum on ascitic forms of rat aud mouse tumors 48 hours after intraperitoneal translantation, especially showing a maked effect on two strains of Hirosaki sarcoma and Ascites hepatoma 130.

1) Mitomycin C showed a marked effect on solid forms of each strain of

Hirosaki sarcoma 3-10 days after subcutaneous transplantation.

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要　旨

Mitomycin C の各種実験腫瘍に対する効果

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Mitomycin C をラッテ，マウスの各種の腫瘍に使用し，次の如き成績を得た。

A) 腹水腫瘍に対する効果：各種の腹水腫瘍を腹腔内移植 48 時間後で純培養に増殖した状態のときに 50～1000 mcg/kg の mitomycin C を毎日腹腔内に注射し，その期間は実験例によってかなりの差異はあるが (3～33 日)，一般に大量投与の場合は短期間，小量投与の場合は長期間におよんだ。実験例は全例生存例百分率曲線によって検討し，その 50% 生存日数が非治療群の 50% 生存日数に比して少くとも 2 倍以上に達したときに延命効果のあるものと判定した。

この結果をまとめてみると，最も著しい効果の認められたものは 2 倍体弘前肉腫であって，50 mcg/kg の連日投与で延命効果が認められた。次で 4 倍体弘前肉腫，肝癌 130 系は 100mcg/kg で延命効果が認められ，マウス Ehrlich 癌は 200mcg/kg で効果が認められた。マウス淋巴性白血病は吉田肉腫とともにかなりの抵抗を示し，500 mcg/kg ではじめて延命効果がえられた。臼淵肉腫は最初の実験では 500 mcg/kg で延命効果がみられたが，その後の増殖力の高い時期には強い抵抗を示し，さらに武田肉腫および肝癌 7974 系は 1000 mcg/kg でも全く効果は認められなかった。この成績は mitomycin C がラッテ・マウスの広範囲の腫瘍に対して強い効果を有することを示すものである。

また，細胞学的には同一系の腫瘍である吉田肉腫と弘前肉腫の間および肝癌 130 系と 7974 系との間に mitomycin C の効果の著しい差違のみられたことは興味深いことである。

B) 皮下腫瘍に対する効果

2 倍体弘前肉腫および 4 倍体弘前肉腫をラッテ皮下に移植し，3～10 日後で皮下腫瘍の明らかな発育を認めてから，腹腔内に mitomycin C 200～500 mcg/kg を連日投与し，7～15 日におよんだ。3 日後に治療開始したものはほとんど全例が治癒し，5～6～10 日後に治療開始したものも，治療期間中に腫瘍は縮少し，治癒するものが多く，一部は治療中止後に再発した。対照非治療群は全例腫瘍死した。この成績は mitomycin C が結節性腫瘍に対しても著明な抑制効果を有することを示すものである。

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STUDIES ON THE MECHANISM OF LIVER-CARCINOGENESIS BY CERTAIN AMINOAZO DYES. I. OVERALL METABOLISM OF DIMETHYLAMINOAZOBENZENE AND CARCINOGENESIS

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INTRODUCTION

It has been reported by Miller and Miller (1, 2) that the chemical binding of certain aminoazobenzene dyes with liver protein(s) in the precancerous stage of the liver of rats fed the carcinogenic aminoazobenzene dyes could be of ethiological significance in the carcinogenesis.

The chemical nature of the dye-protein binding has been investigated using highly purified polar dyes prepared in our laboratory (3, 4). The experimental results obtained up to the present may lead us to assume that the aminoazo dye is bound to tyrosine residue of a protein at the position 3 (ortho position to the amino group of the dye) probably through a methylene bridge which may be derived from formaldehyde produced as a result of the oxidative demethylation. However, the oxidative demethylation is not the only metabolic pathway for the dimethylaminoazobenzene in the rat liver. The reductive cleavage at the azo group of the dye as well as the hydroxylation at the aromatic nucleus can also take place. These reactions proceed simultaneously side by side and a special attention should be paid when the reaction products are analysed in order to discuss the activity of any one of the above metabolic reactions. If DAB is used as a substrate and is incubated with liver homogenate fortified with several cofactors (5), the amount of DAB diminishes in time. The measurement of remaining DAB gives an idea about the overall metabolic activity of the liver including demethylation, hydroxylation and reductive cleavage. Measuring the amount of MAB, or AB produced by the demethylation, however, does not necessarily give any concrete idea about the demethylation activity of the system, because MAB or AB produced from DAB by demethylase can be further modified by hydroxylase or azo-reductase system present in liver. It is clear therefore a strict control to check the side reactions by modifying the reaction system or by selecting an adequate substrate is necessary in order to measure any one of the metabolic activities selectively.

The combination of dyes with liver proteins can take place only through a metabolic reaction of the dye in the liver. Considering the necessity of at least

one N-methyl group of the dye for being carcinogenic, the metabolism of the N-methyl group of the dye is assumed to be at least one of the carcinogenic metabolism in DAB carcinogenesis. The two other metabolic reactions such as hydroxylation and reduction may be called detoxicating metabolism and may not have any direct relation to the carcinogenesis.

The substance which is responsible for binding the aminoazo dye with certain liver protein(s) could be formaldehyde which is a product of oxidation of N-methyl group. The formaldehyde in stat unascendi in the liver cell may be very reactive and contribute to bind the dye molecule with a special protein in the vicinity. The identity of the protein which binds the dye is a very important problem to investigate. It has been demonstrated that the demethylase is located in the microsomal fraction (6, 7) and the dye-protein binding takes place in the microsome at first (8) and then the dye-bound protein is spread into a supernatant fraction as well as other cellular particulate fractions, such as mitochondria and a nucleus in less amount (8). The protein concerned appears to be of specific nature because the amount of the protein-bound dye does not increase beyond a certain upper limit even after a great amount of the dye is administered (9). Increasing the amount of the dye administered at one time or repeating the dye administration with an aid of a stomach tube does not increase the amount of the polar dye beyond the definite upper limit. It is our working hypothesis that the protein may be one of the enzymes which are responsible for the metabolism of the dye.

The current idea, however, concerning the incorporation of the dye into the liver protein supported by Hultin (8) and by Miller et al. (10) is that the dye binding may take place in the microsome in the course of the protein synthesis. They excludes the possibility that the dye can be bound with proteins already present in the cell.

A series of studies have been commenced to investigate these interesting problems. First of all, in this report the general metabolism of DAB and the relation to the carcinogenicity will be discussed. It was confirmed that the narrow specificity concerning the carcinogenicity of DAB such as organ specificity and species specificity may be closely related to its metabolic behavior. It was also demonstrated that the administration of DAB greatly diminished the activity of the liver to metabolize DAB even in a short period after the administration of the dye. It is very interesting to note that the administration of AB, however, did not diminish the enzyme activity.

Experimental

Method and Aparatus:

1) **Animals and Food.** Male albino rats (100-130g weight) were fed rice, the

standard diet (commercial, by the Oriental Yeast Co., Ltd.) or DAB (0.06%) coated rice ad libitum. The kind of food fed to the animals is indicated in every experimental case. Male albino mice (15–20g weight) were also used. A guinea pig purchased from a dealer weighted 800g.

2) **Dye Administration and Enzyme Preparation.** Recrystallised DAB, MAB or AB suspended (partially dissolved) in olive oil or propylene glycol was administered to the rats or mice with a help of a stomach tube under the anesthesia. As a control only a solvent of the same amount was administered. Three days after the administration of the dye, the animals were killed by decapitation and the liver was removed immediately. The type of food given meanwhile is also indicated in every experimental case. The 20% liver homogenate in the physiological saline solution was prepared by a Potter-Elvehjem homogenizer.

3) **Reaction Mixture.** The reaction condition to study the metabolism of the dye was mainly adapted from the paper by Miller et al. (5), except omitting hexosediphosphate from the composition, because the endogenous substrate was proved to be sufficient. The composition of the reaction (11) mixture is as follows:

TPN (raw material, Ca. 1200γ (11))	0.1ml
DPN (commercial, 200γ)	0.1ml
$MgCl_2$ (0.1M)	0.2ml
KCl (0.2M)	0.2ml
Phosphate Buffer (0.1 M, pH 7.5)	1.5ml
Nicotinic Amide (1M)	0.2ml
Water	1.1ml
Enzyme (20 or 10% homogenate)	1.0ml
DAB (about 90γ, in alcohol)	0.1ml (added at the last)
Total Volume:	4.5ml

The above components were mixed in a special Erlenmeyer flask (capacity of 50ml) and the vessel was connected with a Warburg manometer-arm and was shaken gently in a water bath regulated at 37°C for two hours with a cock open to the air.

4) **Extraction of Dyes.** After two hours of incubation, the vessel was detached and two ml of acetone and five ml of benzene were added to stop the reaction The mixture was thoroughly shaken and centrifuged. The upper organic layer was pipetted. The remaining lower layer was stirred up and was extracted with the acetone and benzene mixture as described above. The extraction was repeated again. The three or four extracts were combined and evaporated under reduced pressure. The residue was dissolved in petroleum ether and the petroleum ether solution was submitted to the aluminum chromatography.

5) **Separation of the Metabolic Products.** The Brockmann alumina column (0.7×10cm) was used. As the first eluting solvent a mixture of benzene and

petroleum ether (1 : 2) was used. DAB ("a" band) was eluted first. MAB ("b" band) following DAB could be eluted as the second band. After MAB was fractionated the solvent was switched to a mixture of benzene and petroleum ether (2 : 1). AB ("c" band) could be fractionated as the third band. After collecting AB, the yellow band remaining at the top of the column was then eluted with acetone. Usually two bands, the first, swiftly moving and the second, slowly moving bands were named band "d" and band "e", respectively. The still unmovable yellow band at the top of the column could be eluted with 30% alcoholic 0.01 N HCl and was named as band "f". It appeared that the last, "f" band could be a mixture and may be further fractionated. The first three bands, a, b and c bands, were identified as DAB, MAB and AB from the spectrometric analysis. The amounts were also calculated from the optical density measurement in acid media at 520, 510 and 500mμ, respectively. The last three bands, d, e and f, which have absorption maxima at 540~545mμ in acid medium were still not identified but considered to be hydroxylated aminoazobenzene derivatives.

Results :

The ability of liver to metabolize DAB differs depending upon age, sex, nutritional or other physical conditions of the experimental animals as well as the reaction conditions. In the case of studying the effect of dye administration upon the ability to metabolize DAB, a control experiment was always carried out using the animals having the same biological condition. It seems therefore rather difficult to compare the experimental results obtained with the animals cared under the different conditions. TPN used in this study was a partially purified material and contained a great deal of insoluble substance. The net concentration of TPN in the experiments reported in this paper could be more or less fluctuated. Since the animals were not kept in a constant temperature room, they appeared to loose the appetite as the temperature got up. The decreased metabolic activity was also observed in paralell to this seasonal temperature rise.

1) Comparison of DAB-Metabolizing Activity of Livers of Rat, Mouse and Guinea Pig.

Among the experimental animals only the rat is known sensitive to DAB carcinogenesis. Hepatoma is rarely induced in mice, but not in any other animals. In order to investigate the reason the DAB-metabolizing ability of livers of rats, mice and a guinea pig were compared under the same conditions. In this experiment, only the amounts of DAB, MAB and AB were measured. The amounts of these aminoazo dyes after two hours of incubation are listed in Table 1.

It is clear that the overall metabolism of DAB is the most active with rats liver. The ability for the liver of mice or guinea pig to metabolize DAB is far behind that of rat liver. It should be mentioned here that the amount of MAB

or AB obtained after the incubation of DAB with liver homogenate can not be a measure to show the demethylase activity of the homogenate, because MAB or AB produced by the demethylase can further be hydroxylated or reduced during the incubation, thus diminishing the amount to be recovered. This point seems to have been neglected in the experiments mostly performed by Miller et al. to discuss the demethylase activity.

The decrease in the amount of DAB indicates the overall metabolic activity, and the difference between DAB added and the sum of DAB+MAB+AB recovered indicates the combind ability of hydroxylation and azo reduction.

Table 1. Comparison of DAB-Metabolizing Activity of Livers of Rat, Mouse and Guinea Pig.

Animal	Conc. of Homogen. added to the react. mixture (%)	Dyes Separated				
		Enzyme dry wt. (g)	a(DAB) (γ)	b(MAB) (γ)	c(AB) (γ)	a+b+c (γ)
Guinea Pig	20	0.0481	37.8	12.3	2.2	52.3
	10	0.0240	65.3	9.6	1.0	75.9
	0	0	84.6	0.6	0.0	85.2
Mice	20	0.0523	32.0	4.7	1.7	38.4
	20	0.0523	32.9	5.2	1.3	39.4
	10	0.0261	54.0	11.2	2.6	67.8
	10	0.0261	53.1	11.5	4.0	68.6
Rats	20	0.0520	3.1	3.8	0.4	7.3
	20	0.0520	2.4	0.4	0.2	3.0
	10	0.0260	12.3	0.9	0.4	14.1
	10	0.0260	12.3	0.8	0.8	13.9

All the animals were fed the standard diet for at least three days before the assay.

2) Comparison of DAB-Metabolizing Ability of Liver, Kidney, Testis and Spleen of Rats. Conney et al. (7) have shown that rat liver homogenate yielded a great amount of 3-Me-AB, out of 3-Me-MAB, but kidney, spleen, brain, lung, heart, and small intestine and liver tumor failed to demethylate the substrate with ease. Liver is known only one organ which is sensitive to the DAB carcinogenesis. The 20% homogenate of testis, kidney, spleen and liver from two young rats (100g) fed rice for three days in advance were prepared immediately after sacrficing the animals. The reaction and the assay were carried out as described above. The results are shown in Table 2.

Comparing with the high metabolic activity of the liver, the spleen and the kidney showed only a weak ability to metabolize DAB. The testis seems to be almost inactive.

It is very interesting to find that the only organ which is sensitive to the DAB

Table 2. Comparison of DAB-Metabolizing Activity of Liver, Kidney, Testis and Spleen of Rats.

Organ	Dyes Separated					
	a(DAB) (γ)	b(MAB) (γ)	c(AB) (γ)	a+b+c (γ)	d(o.d. x ml)	e(o.d. x ml)
Testis	23.2	1.5	0.9	85.6	0.020	—
Kidney	74.3	3.6	0.7	78.6	0.015	—
Spleen	71.1	1.3	0.6	73.0	0.030	—
Liver	46.8	6.7	7.7	61.2	0.580	0.340

The rats were fed rice for three days before the assay. o.d. is optical density.

carcinogenesis is the highest in the DAB metabolism. The same principle was also shown to be applied to the species specificity. It is therefore clear that the metabolism of DAB is prerequisite for the DAB carcinogenesis.

3) Effect of Dye Administration upon the DAB-Metabolism of Liver.

Miller et al. (12) have shown that feeding of 3'-Me-DAB considerably depletes the demethylase activity in rats liver. In the liver tumor induced by the carcinogen, the demethylase is completely depleted (7, 12). It is the author's hypothesis that the protein binding with the aminoazo dye in liver could be one (or some) of the enzymes related to the metabolism of the dye. It has been reported (13) that the dye-protein binding reaches a maximum in three days after the administration of a large dose of DAB (20–50mg) with a help of a stomach tube. The experiments were carried out to investigate the effect of the dye administration upon the metabolic ability of rats or mice liver against DAB.

A certain amount of dye was administered with an aid of the stomach tube, and the liver was removed three days after the administration of the dye. The result is shown in Table 3. As shown in Table 3, the administration of DAB to

Table 3. Effect of Dye Administration upon DAB-Metabolism of Liver.

A: Rats.

Dye	Solvent	Conc. of homogen. (%)	Food (3 days)	Dyes Separated						
				a (DAB) (γ)	b (MAB) (γ)	c (AB) (γ)	a+b+c (γ)	b (o.d. x ml)	e (o.d. x ml)	f (o.d. x ml)
DAB (33.3 mg)	Prop.*** (2 ml)	20	DAB-rice*	71.1	7.7	2.6	80.8	—	—	—
None	Prop. (2 ml)	20	Rice	32.3	0.9	0.7	34.4	—	—	—
DAB (33.3 mg)	Prop. (2 ml)	10	DAB-rice	78.8	6.3	2.1	87.2	—	—	—
None	Prop. (2 ml)	10	Rice	65.3	3.1	2.1	70.5	—	—	—

MAB (50 mg)	Prop. (2 ml)	20	DAB-rice	34.7	17.5	17.9	70.1	—	—	—
MAB (25 mg)	Prop. (1 ml)	20	DAB-rice	34.6	15.7	10.7	61.0	—	—	—
MAB (50 mg)	Prop. (2 ml)	10	DAB-rice	45.0	13.9	10.5	69.4	—	—	—
MAB (25 mg)	Prop. (1 ml)	10	DAB-rice	54.0	14.7	4.5	73.2	—	—	—
DAB (50 mg)	Oliv.**** (1 ml)	20	DAB-rice	68.4	4.9	1.4	74.7	0.180	0.160	—
None	Oliv. (1 ml)	20	Rice	36.9	7.9	4.4	49.2	0.595	0.305	—
DAB (50 mg)	Oliv. (1 ml)	10	DAB-rice	64.3	5.0	1.4	70.7	0.160	0.210	—
None	Oliv. (1 ml)	10	Rice	31.5	8.9	0.7	41.1	0.430	0.265	—
AB (50 mg)	Oliv. (1 ml)	20	Rice	40.5	3.5	1.8	45.8	0.125	0.240	—
None	Oliv. (1 ml)	20	Rice	57.2	3.6	0.9	61.7	0.140	0.040	—
None	Oliv. (1 ml)	20	Stand.**	31.5	5.8	1.4	38.7	0.305	0.120	—

* DAB (0.06%) coated rice
** Standard Diet
*** Prop. = Propylene glycole
**** Oliv. = Olive oil

B: Mice.

Dye	Solvent	Conc. of homogen. (%)	Food (3 days)	Dyes Separated						
				a (DAB) (γ)	b (MAB) (γ)	c (AB) (γ)	a+b +c (γ)	d (o.d. x ml)	e (o.d. x ml)	f (o.d, x ml)
DAB (3.3mg)	Prop. (0.2 ml)	20	DAB-rice	62.6	8.4	2.7	73.7	—	—	—
None	Prop. (0.2 ml)	20	Rice	64.4	4.5	0.9	69.8	—	—	—
DAB (3.3mg)	Prop. (0.2 ml)	10	DAB-rice	63.9	12.5	3.1	79.5	—	—	—
None	Prop. (0.2 ml)	10	Rice	68.9	7.8	1.3	78.0	—	—	—
DAB (10mg)	Oliv. (0.2ml)	20	DAB-rice	78.0	5.2	2.1	85.3	0.060	—	—
DAB (10mg)	Oliv. (0.2ml)	20	DAB-rice	77.0	4.3	1.6	82.9	0.020	—	—

rats caused the remarkable decrease in the hepatic ability to metabolize DAB. The administration of MAB or AB does not seem to reduce the activity, but a little stimulating effect was observed, especially with AB.

The administration of DAB to mice does not show any clear-cut inhibitory effect upon the DAB metabolism as observed with rats.

C: Effect of Long Feeding of Rats on DAB-Rice Diet.

Food	Period (days)	Conc. of homogen. (%)	Dyes Separated						
			a (DAB) (γ)	b (MAB) (γ)	c (AB) (γ)	a+b+c (γ)	d (o. d. x ml)	e (o. d. x ml)	f (o. d. x ml)
Stand.	7	20	2.2	1.2	28.6	32.0	1.58	0.83	0.49
Stand.	7	10	16.6	5.1	21.9	43.6	1.22	0.68	0.01
DAB-rice	35	20	23.9	6.8	16.2	46.9	1.15	0.57	0.27
DAB-rice	171	20	64.5	8.5	6.9	79.8	0.44	0.25	0.15

As long as DAB is used as the substrate, we can not discuss any one of the metabolic pathways available to DAB separately, but the overall metabolism can only be taken up. By selecting a proper dye able to be used as a specific substrate, we may be able to solve this complexity It is very interesting to find that there is a close relationship between the carcinogenicity of the aminoazodye and the effect of its administration upon the hepatic activity to metabolize the dye.

Summary

1) A method to fractionate the metabolic intermediates of DAB after the dye was incubated with the fortified liver homogenate, has been established.

2) It has been demonstrated that at least three metabolic pathways are available under the experimental conditions: demethylation, hydroxylation and reductive cleavage of the azo bond.

3) The species specificity and the organ specificity concerning the DAB carcinogenesis was shown to be related to the metabolism of the dye. DAB was metabolized by rat liver with the highest speed but not by other animal's liver or other organs of rats.

4) A large dose of DAB administered with the aid of a stomach tube caused a remarkable decrease in the ability of rats liver to metablize DAB even three days after the administration. The administration of DAB, however, did not change the activity of mice liver much. Administration of MAB or AB seems to have no effect.

5) The above observations do not contradict our working hypothesis that the carcinogenic aminoazo dye might be bound with one (or some) of the enzymes in microsomes responsible for metabolizing the dye administered.

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要　旨

アミノアゾ色素による発癌の機構に関する研究
第一報　ヂメチルアミノアゾベンゼン
の代謝と発癌性の関係について

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1) ヂメチルアミノアゾベンゼン (DAB) の肝ホモヂェネートによる代謝産物をクロマト的に分離定量する方法を研究した。これによって反応液中より DAB, MAB, AB のみならず少くとも3種の水酸化アミノアゾベンゼン誘導体が分離された。

2) モルモット，ラッテ，およびマウスの肝臓において，DAB の全体的な代謝能力を比較したところ，ラッテ肝が圧倒的に強い活性を示すことが分った。

3) ラッテの肝臓，腎臓，睾丸，および脾臓について，DAB に対する代謝能力を比較したところ，肝臓が最も強力で腎臓脾臓は極めて弱く，睾丸はほとんど不活性であった。

4) DAB, MAB または AB を予めゾンデでラッテの胃に一定量 (20～50mg) 投与すると，DAB の場合には極めて顕著な DAB 代謝能力の低下が，色素投与後3日目の肝臓について観測された。興味あることは発癌力のない AB についてはこのような活性の低下は全く認められないことである。マウスに DAB を投与した際の影響は，ラッテについてみられたような顕著なものは認められないようである。

5) 以上のような実験的事実は，DAB の発癌にその代謝が重要な条件となっていることを意味するものであって，DAB が肝臓内で代謝される段階において，発癌に関係する機構が結びついていることを示すといえよう。DAB の脱メチル化がこの際特に問題になるのは AB のようなものでは影響が全く現れないことからも考えられることである。in vivo においてアミノアゾ色素と結合する相手の蛋白質が何であるかわなお不明であるが，われわれはこれに対して，DAB の代謝に何等か関係をもっている酵素であろうと想像しているのであって，現在のところこれに矛盾するような事実には遭遇していない。

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